FEEDBACK INHIBITION OF CATABOLIC PATHWAYS

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FEEDBACK INHIBITION OF

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CHAPTER I

INTRODUCTION

Enzymes of certain catabolic pathways which are synthesized in significant amounts only in the presence of specific inducer molecules have been termed "inducible enzymes" (Monod and Cohn, 1952). The inducer molecule (the substrate) interacts with the product of a regulator gene (the repressor), thus allowing enzyme formation (Jacob and Monod, 1961). Other enzymes, which are produced in fixed amounts, independent of need, are called constitutive enzymes. It is found that some carbon sources such as glucose, which are fermented by constitutive enzymes, are readily metabolized no matter what medium is used for prior growth of the bacteria.

The ability of glucose to inhibit the synthesis of certain enzymes required for the utilization of other sugars is termed the "glucose effect". This effect is not entirely specific for glucose. Depending on the organism, compounds closely related to glucose such as gluconic acid, mannitol or galactose, which can serve as a ready source of metabolic intermediates and energy, may be equal to glucose in their inhibitory effect on the formation of glucose-sensitive enzymes (Magasanik, 1961). Glucose is generally more rapidly metabolized than other carbon compounds. This is due to the fact that cells grown on glucose possess, in addition to the constitutive series of enzymes responsible for the degradation of glucose via triose phosphate to pyruvate, another induci-

ble series of enzymes which catalyze the rapid dissimilation of glucose via gluconic acid (Magasanik, et al. 1959).

In the glucose effect, glucose itself is not the functional repressor (Magasanik, et al., 1958), but is converted to other substances that, possibly upon "activation", cause the various repressions.

Two types of mechanisms have been generally cited as the major controls for regulating the flow of metabolites through metabolic pathways. These two mechanisms are referred to as "repression" and "feedback inhibition". Repression has been defined as a relative decrease, resulting from the exposure of cells to a given substance, in the rate of synthesis of a particular apoenzyme (Vogel, 1957). Feedback inhibition occurs when the accumulation of the end product of a biosynthetic reaction sequence brings about a rapid inhibition of enzymes operating specifically to produce that product. The difference between "repression" and "feedback inhibition" primarily lies in the fact that the former involves the inhibition of enzyme synthesis and hence controls the amount of enzymes produced, whereas the latter involves the immediate control of enzyme activity.

Magasanik (1961) favored the hypothesis that glucose, because it is catabolized faster than other substrates, produces higher levels of metabolic intermediates, one or more of which then act as a repressor or a precursor of a repressor inhibiting the synthesis of inducible enzymes. He suggested the term "catabolite repression". Catabolite repression of the lactose operon of Escherichia coli is thought to be due to a gene, CR, which determines an inactive repressor (Loomis and Magasanik, 1967a). This repressor is presumably activated by one or more catabolites and when activated, is able to interact with the opera-

tor gene and thereby inhibit transcription of the structural genes.

Cohen and Monod (1957) suggested that the enzyme-like factors (named permeases), that permit entry and control concentration within the cells, must play a role in metabolic regulation. Entry of a nutrient into the cell is generally the first step in its metabolism. Permeases make available at high intracellular concentrations nutrients present at low concentrations in the environment and thereby permit the nutrients to be metabolized rapidly by a limited amount of enzyme. (Pardee, 1960).

Recently Loomis and Magasanik (1967b) have demonstrated that glucose inhibits uptake of lactose by glucose-grown CR^+ cells. This effect can be overcome by preinduction with the inducer methyl thiogalactoside or by high concentrations of lactose. The primary cause of glucose-lactose diauxie is inhibition of inducer transport rather than catabolite repression. Adhya and Echols (1966) had demonstrated a similar glucose inhibition of inducer transport for the galactose operon of Escherichia coli and concluded that the primary mechanism of glucose control in this case is inhibition of inducer transport rather than catabolite repression.

Kornberg and his associates (1960) had reported that the utilization of the glyoxylate cycle by Micrococcus denitrificans was governed largely through a combination of feedback inhibition and repression mechanisms. MacQuillan and Halvorson (1962) had found that glucose at low concentrations showed a stimulatory effect on β-glucosidase synthesis, whereas at high concentrations it caused repression of such enzyme synthesis. Gorini and Maas (1957) had reported that the level of concentration of an inhibitor required for maximal feedback inhibition

seems to be lower than that needed to give maximal repression. Hence, the levels of inhibitor concentration also seem to be a significant factor in regulating these control mechanisms.

Gaudy (1962a) studied induction and repression in activated sludge systems. Diauxic substrate removal by activated sludge was observed in a minimal medium containing glucose and sorbitol as carbon sources. The data showed the removal of glucose before metabolism of sorbitol although the population was acclimated to sorbitol. This indicated that the prevention of sorbitol removal by glucose could be caused by interference with the functioning of the enzyme system required to metabolize sorbitol as well as by repression of enzyme synthesis. Gaudy, Gaudy and Komolrit (1963) reported that glucose acted as a suppressor of sorbitol metabolism, when large initial inocula of sorbitol-grown cells (either Escherichia coli or a heterogeneous population) were employed. Since a fairly large initial cell concentration was used and these cells already possessed a functioning system for sorbitol metabolism, its elimination from the medium would not depend entirely upon the synthesis of new enzymes. Glucose and sorbitol were also shown to be removed sequentially in an experiment with cells acclimated to sorbitol when both carbon sources were present at very low concentrations. The data indicated that glucose was removed first (Bhatla and Gaudy, 1965). These results supported the conclusion that sequential removal of glucose and sorbitol did not depend only upon the repression of enzyme formation by glucose, but that glucose could also suppress the functioning of existing enzymes involved in sorbitol-metabolizing pathways.

Besides the sorbitol and glucose substrate system, a large number of multicomponent systems has been studied by Gaudy and his coworkers

(Gaudy, Komolrit and Gaudy, 1964; Komolrit and Gaudy, 1964; Su, 1968). In these studies, it was found in a number of cases that the utilization of a carbon source may be immediately blocked if a second carbon source is added to the medium, even though the cells had been previously acclimated to the former carbon source and thus should possess functioning enzyme systems required for its utilization. Gaudy has reasoned that this rapid control would appear to be possible only through immediate inhibition of the function of the enzymes and could be analogous to feedback inhibition of a biosynthetic pathway. By analogy with metabolite repression, it was postulated that such control acts through accumulation of common intermediates, favoring utilization of the more rapidly metabolizable carbon source, and would possibly function only for combinations of carbon sources which do produce common intermediates.

Gaudy, Komolrit and Bhatla (1963) had indicated that the physiological condition operationally defined as "sludge age" plays an important role in controlling the extent of the inhibitory effect. For "old" sorbitol-acclimated sludge, sequential removal did not occur and glucose metabolism was rather slow. A mannitol-acclimated sludge showed no utilization of glucose by old cells (Gaudy, Komolrit and Gaudy, 1964). In these two systems, it is possible that an initial enzyme step required to bring glucose into the Embden- Meyerhof pathway or glucose permease, required by the cells for glucose uptake, was absent in the old cell sludge and had to be induced. A decrease in the rate at which glucose can be metabolized by old cells might prevent the accumulation of an intermediate necessary for operation of the control mechanism. An increase in the age of the sludge is also accompanied by a decrease in the rate of substrate removal, and a longer acclimation period is re-

quired to metabolize new compounds.

Krishnan and Gaudy (1965) reported that utilization of glycerol by glycerol-acclimated cells was greatly reduced in the presence of glucose. Recently, Zwaig and Lin (1966) have also reported feedback inhibition of the glycerol pathway in a mixture of glucose and glycerol. The utilization of glycerol by cells of Escherichia coli had been shown to be obligatorily dependent upon glycerol kinase (Lin et al., 1962) which not only mediated the first reaction in the pathway for the dissimilation of glycerol, but was also responsible for the trapping of the substrate diffused freely across the cell membrane (Hayashi and Lin, 1965). In attempting to isolate mutants in which the formation of glycerol kinase was resistant to glucose repression, a strain was found which was able to incorporate glycerol while growing on glucose. Zwaig and Lin (1966) suggested that there is another type of control mechanism in addition to catabolite repression. Experimental data showed the control was effected through a kinetic feedback mechanism. The cells grown on glucose were replete in an intermediate which inhibited severely the glycerol metabolism of wild-type cells but not that of the mutant cells. That the difference in behavior between the two types of cells reflected the properties of their respective glycerol kinases rather than enzymes further on in the pathway was indicated by the finding that the kinase of the mutant was inactivated at pH 9.5, a condition under which the activity of the wild type enzyme is nearly maximum.

In an attempt to identify the inhibitor, several glycolytic intermediates were tested for their effects on the activity of glycerol kinase. This was the first in vitro study on catabolite feedback inhibition. They found that fructose-1, 6-diphosphate was inhibitory. The

activity of the kinase was a function of the concentration of fructose-1, 6-diphosphate and was independent of glycerol concentration. Only the rate of catalysis and not the substrate affinity of the enzyme was regulated by the effector, thus making the kinase a "V" allosteric enzyme (Monod, Wyman and Changeux, 1965).

From the experimental data, fructose-1, 6-diphosphate caused a fourfold reduction in the activity of the wild type enzyme while virtually no inhibition occurred with the mutant enzyme. Further evidence for this regulatory role of fructose-1, 6-diphosphate was provided by Bŏck and Neidhardt (1966a, 1966b), in their study of an Escherichia coli mutant possessing a temperature-sensitive fructose-diphosphate aldolase which was able to grow in rich medium at 30°C but not at 40°C. This mutant was used to study the physiological effect of a specific block in the Embden-Meyerhof glycolytic path. Growth of the mutant at 40°C was found to be inhibited by the presence of glucose or certain related compounds in the medium. At 40°C glucose was metabolized at 30 to 40% of the control rate and the metabolism was abnormal in that glucose was converted into other six-carbon substances such as gluconate. These were excreted into the medium; then the excreted metabolites were slowly taken up and completely oxidized presumably via the hexosemonophosphate pathway. Throughout the stage of glucose metabolism at 40°C, the fructose-1, 6-diphosphate pool was maintained at a level more than 20-fold above normal. It would appear that the accumulation of fructose-1, 6-diphosphate is somehow a cause of the sensitivity of these of cells to glucose at 40°C.

When glycerol was used to grow the mutant at 40°C, the growth inhibition by glucose was accompanied by cessation of glycerol metabo-

lism. Growth on α-glycerol phosphate was not inhibited under these conditions, implicating glycerol kinase as a possible site of inhibition. Böck and Neidhardt stated that severe inhibition of growth by a substance whose catabolism is incomplete because of a mutationally or chemically blocked reaction has by now become a fairly common observation. They concluded that the inhibitory effect of glucose on growth at 40°C was not caused by a deficit of intracellular adenosine triphosphate, but might be the result of a generalized poisoning of many cell processes by a greatly increased intracellular concentration of fructose-1, 6-diphosphate.

Previously, sequential substrate utilization has been studied primarily with heterogeneous populations. Only two papers (Gaudy, Gaudy and Komolrit, 1962; Gaudy, Komolrit, Gaudy and Bhatla, 1963) reported sequential substrate removal by a pure culture of Escherichia coli.

Both Zwaig and Lin (1966) and Böck and Neidhardt (1966) failed to suggest a general significance for their results. Zwaig and Lin discussed the advantages of a mechanism of inhibition for the glycerol pathway specifically. Böck and Neidhardt attributed their results to a generalized organic phosphate poisoning, while Zwaig and Lin interpreted the specific inhibition of glycerol kinase as a mechanism to prevent accumulation of toxic levels of phosphorylated compound. In neither paper was the possibility mentioned that feedback inhibition of degradative pathways may be a control mechanism of general importance. However, studies reported by Gaudy and co-workers using heterogeneous populations and various combinations of substrates indicate that this mechanism is not confined to a few species of microorganisms or to the glycerol pathway. The present study was undertaken to determine the extent of

occurrence of feedback inhibition (or sequential substrate removal) in pure culture systems using several combinations of carbon sources. Experiments were designed to determine whether "cell age", reported to influence the response of a heterogeneous population, would have a similar effect with a pure culture. A third objective of these studies was to obtain data relative to the mechanism of the inhibition.

CHAPTER II

MATERIALS AND METHODS

Organisms

Escherichia coli strain 45 was obtained from the stock culture collection of the Department of Microbiology, Oklahoma State University. *Achromobacter* sp. was isolated from sewage in minimal medium with sorbitol as carbon source by P. Krishnan of the Bioengineering Laboratories, Oklahoma State University. *Escherichia coli* strain W3110 was obtained from Dr. M. L. Morse, University of Colorado.

Cultivation Media and Conditions of Growth

Flask Cultures

1. Young Cells

The cells were grown at 37°C in a minimal medium of M-9 salts (Roberts, et al., 1957) containing (in grams per liter): NH_4Cl, 1.0; $Na_2HPO_4 \cdot 7H_2O$, 11.3; KH_2PO_4, 3.0; NaCl, 5.0; $MgSO_4 \cdot 7H_2O$, 0.2 and distilled water. Carbon sources used included sorbitol, glucose, galactose, glycerol, ribose, arabinose, histidine and raffinose. These were autoclaved or filter-sterilized separately at a concentration of 10 per cent (W/V) and added to the desired concentration. Yeast extract was sterilized separately at a concentration of 2 per cent (W/V) and added as needed.

The cells were grown at 37°C on a reciprocal shaker overnight. Cells were harvested by centrifugation (10 min, 10,000 rpm Servall, RC2-B), resuspended with M-9 medium and transferred to flasks of freshly prepared growth medium containing the appropriate carbon sources. The flasks were placed on a shaker at 37°C and samples were withdrawn for measurement of substrate removal at various time intervals. Growth was measured by determining optical density at 540 mμ in the test tube side arm of the flask. Details of experimental procedure are given for each experiment.

2. Old Cells

In order to have enough cells for the experiment, six slants were inoculated. After they grew overnight in the 37°C incubator, the cells were transferred to 12 flasks with the synthetic growth medium of the same composition as that described above using 0.1 per cent sorbitol as a carbon source and placed on a shaker at 37°C. Each day two flasks of cells were harvested and resuspended for use in a substrate removal experiment. From the rest of the flasks, 2 ml of culture were removed each day and substituted with 2 ml of 10 per cent sorbitol. Another set of experiments similar to the above was run except 2.2 ml of culture were removed and replaced with 2 ml of 10 per cent sorbitol and 0.2 ml of 2.0 per cent yeast extract.

Continuous Flow System

The apparatus used was fabricated wholly in glass. The design is shown in Figure 1. A 4-liter flask was used for storing medium containing 0.2 per cent sorbitol as carbon source to be fed into the chemostat.

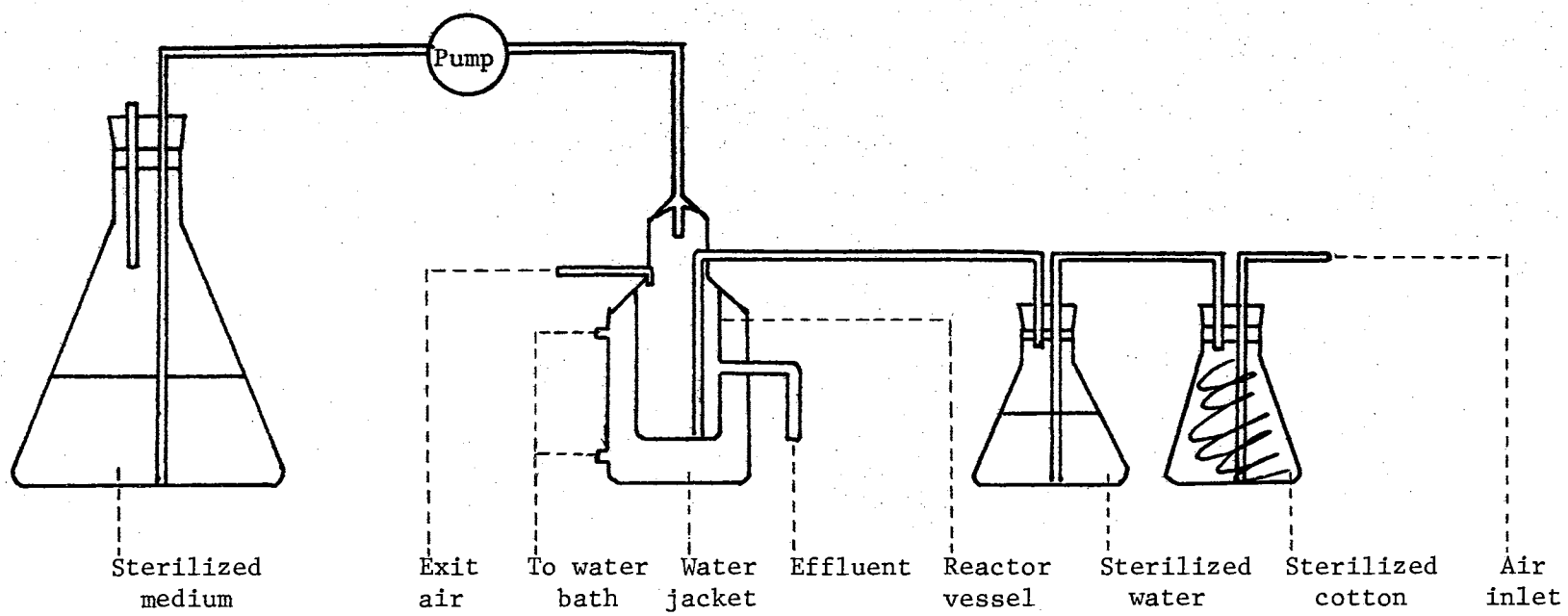


Figure 1. Schematic Drawing of the Continuous Flow System

The feed line to the system was regulated by a liquid flow meter pump (Sigmamotor, Inc., Middleport, N. Y.) for which the discharge volume could be set at a definite rate. The aeration and mixing were effected by an air line passed through a flask filled with sterile cotton to filter the air and through sterilized water to saturate the air with water. The temperature of the system was maintained at 25°C by using a thermostatically controlled constant temperature water bath. A pump circulated water from the bath through the jacket surrounding the culture vessel. To initiate operation, cells were grown in 50 ml of M-9 medium with 0.2 per cent sorbitol on a shaker at 37°C overnight. This culture was transferred into the chemostat which was then filled to the overflow line with medium. Aeration was started, the temperature was adjusted, and the cells were allowed to grow for several hours before pumping of medium was started. The medium used was carbon-limited.

Measurement of Growth

Cell suspensions were read against a water blank at 540 mμ on a Coleman Junior Spectrophotometer, Model 6-D.

Chemicals

Dextrose, d-sorbitol and glycerol were obtained from Fisher Scientific Company. Raffinose and l-arabinose were products of Difco Laboratories. Histidine·HCl, C. P. (monohydrate) was obtained from Nutritional Biochemicals Corporation. Galactose was from either Difco Laboratories or Fisher Scientific Company. D-(-)-ribose was from Eastman Kodak Company. Glucostat and Galactostat reagents were obtained from Worthington Biochemical Corporation.

Chemical Analysis

Glucose

To 9 ml of prepared Glucostat reagent, a one ml sample containing 0.05 to 0.3 mg glucose was added and the tube was allowed to stand at room temperature for exactly 10 minutes. One drop of 4 M HCl was added to stop the reaction and stabilize color. Tubes were allowed to stand for five minutes after stopping the reaction, then absorbance was read on a Coleman Junior Spectrophotometer at 400 mμ.

Galactose

Two ml prepared Galactostat reagent were added to 2 ml of sample containing 25 to 100 μg of galactose. Tubes were shaken and incubated on a shaker at 37°C for one hour. After one hour, 6 ml of 0.25 M glycine buffer, pH 9.7, were added to stop the reaction and stabilize the color. Absorbance was read at 425 mμ.

Polyalcohol Determinations for Sorbitol and Glycerol

Sorbitol and glycerol were determined using Komolrit's modification (1965) of the method of Neish (1952).

Polyalcohols can be oxidized by periodate in an acid solution to form formaldehyde. The amount of formaldehyde produced can be determined colorimetrically. To a 2 ml sample, containing 0.05 to 0.3 mg of sorbitol or 0.025 mg to 0.2 mg of glycerol, were added 0.1 ml of 10 N H_2SO_4 and 0.5 ml of freshly prepared 0.1 M periodic acid. Exactly ten minutes later, 0.5 ml of 1 M, freshly prepared, sodium arsenite was added and mixed well. After waiting about 10 minutes, 6.9 ml of abso-

lute alcohol were added with complete mixing. A 1.0 ml sample of the mixture was transferred into another test tube, 10 ml of chromotropic acid reagent (60% H_2SO_4 containing chromotropic acid at 0.1 per cent) were added, and the tube was heated in a boiling water bath for 30 minutes under diffused light. After cooling to room temperature, the color was read at 570 mμ.

Anthrone Test for Total Carbohydrates

Carbohydrates were determined according to Gaudy (1962b). A sample containing 0.05 to 0.15 mg of carbohydrate was made up to 3.0 ml with distilled water. Tubes were cooled in an ice water bath (5°C) for at least one hour and then 9 ml of ice-cold, freshly prepared anthrone reagent, 0.2 per cent anthrone (W/V) in 95 per cent H_2SO_4, were added and mixed thoroughly. Tubes were heated in a boiling water bath for exactly 15 minutes. The tubes were cooled to room temperature and the color was read at 540 mμ.

Nelson's Test for Reducing Sugar

Reducing sugar was determined by the method described by Nelson (1944) which is based on reduction of Cu^{++} in alkaline solution by free reducing groups in a carbohydrate. One ml of sample containing 100 μg/ml reducing sugar was added to 1 ml of Nelson's alkaline copper reagent. The tubes were heated in a boiling water bath for exactly 20 minutes and then cooled to room temperature. One ml of arsenomolybdate reagent was added to each tube to dissolve the precipitated Cu_2O and to reduce the arsenomolybdate. After the Cu_2O had dissolved, 7.0 ml of distilled water were added, the tubes were mixed well and the optical den-

sity was read at 540 mμ.

Orcinol Test for Pentoses

Quantitative analysis for pentoses was described by Mejbaum (1953). Six ml of acid reagent, which was prepared by adding 2 ml of 10 per cent $FeCl_3 \cdot 6H_2O$ to 400 ml of concentrated HCl, and 0.4 ml of 6 per cent orcinol in 95 per cent ethanol were added to 3 ml of sample containing 5 to 25 μg of pentose. The tubes were heated in a boiling water bath for 20 minutes, then cooled to room temperature, and the optical density read at 660 mμ.

Histidine

Histidine was determined according to the method of Jorpes (1932) by reacting iminazole with diazobenzene sulphonic acid. A diazonium solution was prepared by combining 1.5 ml of 5 per cent sodium nitrate solution with 1.5 ml of a solution containing 0.9 per cent sulphonic acid and 9 per cent HCl. The mixture was cooled in ice for 5 minutes and then 6 ml of the nitrate solution were added with shaking. After cooling for 6 minutes, water was added to a volume of 50 ml. One ml of a neutral to faintly acid histidine solution containing 0.05 to 0.005 mg histidine was combined with 2 ml of diazonium solution. After 1 to 3 hours, 5.0 ml of 1.1 per cent sodium carbonate solution were added. The reading was made 4 to 8 minutes after the addition of carbonate solution at 500 mμ.

Differentiation of Substrates in Mixtures

Glucose-sorbitol

Glucose was determined by the Glucostat method described above, which is specific for glucose. Since glucose also reacts with periodate, but at a much slower rate than does sorbitol, it was necessary to correct the measurements of periodate-positive material for the amounts of glucose actually present, using a standard curve for glucose measured with periodate. Sorbitol concentration was thus obtained by subtracting the polyalcohol equivalent of the glucose in the sample from the total determined using the polyalcohol method described above.

Glucose-glycerol

Glycerol concentration was determined by using the polyalcohol method and corrected for the amount of glucose in the sample as described for sorbitol.

Glucose-arabinose and Glucose-ribose

Arabinose and ribose were determined by the orcinol test described above and glucose by the Glucostat method. Neither sugar interferes in the method used for determination of the other.

Glucose-histidine

Histidine concentration was measured by the method of Jorpes (1932) described above and glucose by the Glucostat method. Neither compound interferes with measurement of the other.

Galactose-sorbitol

Galactose was determined by the Galactostat method described above and sorbitol by the periodate method for polyalcohols. Sorbitol measure-

ments were corrected for the amounts of galactose present as described for the glucose-sorbitol mixture.

Galactose-ribose

Both galactose and ribose concentrations were determined by the methods described above. Neither compound interferes with measurement of the other.

Galactose-raffinose

Because the Galactostat reagent could react with both galactose and raffinose, galactose was determined by Nelson's test for reducing sugar above. Raffinose is a non-reducing trisaccharide and is not detected by this method. Total carbohydrates were determined by the anthrone method and raffinose concentrations were calculated by subtracting the amounts of galactose present.

CHAPTER III

RESULTS

To simplify the presentation of the experimental results, all curves representing the inhibition studies are designated by the first letters in the names of the compounds employed in that particular experiment, e.g. if glucose was used to inhibit a system metabolizing sorbitol, the figure is labelled S + G_o, where G_o means glucose was added at zero time.

The utilization of substrates in all the figures is presented as percentage of the initial concentration remaining.

Optical density readings were not plotted. Initial and final optical density readings for all flasks are presented in tables following each group of experiments.

For all experiments, the amount of the second carbon source added to the flasks was the same for each flask in the experiment; i.e., the amount was not decreased to compensate for the relatively small decrease in volume due to removal of samples. Final concentrations given for individual experiments are those for the flask which received both substrates at zero time.

Flask Cultures

Inhibition by Glucose of Utilization of Various Sugars by Young Cells of Escherichia coli 45

To test the effect of glucose on sorbitol-adapted E. coli 45, cells were grown on 1.0 per cent sorbitol M-9 medium on a shaker at 37°C for two days. One ml was transferred to fresh M-9 medium with 0.2 per cent sorbitol and grown on the shaker overnight. The culture was harvested by centrifugation, resuspended in M-9 salts and divided into 4 flasks. One flask contained 0.2 per cent glucose (the glucose control), and the rest contained 0.2 per cent sorbitol. One was used as the sorbitol control and to the other two glucose was added to a concentration of 0.1 per cent at zero time and after two hours, respectively. All flasks were aerated at 37°C on the shaker and at one-hour intervals a sample was removed from each flask to determine the substrate concentrations.

The data are shown in Figure 2. It may be seen that sorbitol-grown cells utilize glucose more rapidly than they utilize sorbitol. In the controls, glucose was depleted in 7 hours while sorbitol had only been reduced by 45 per cent. Figure 2a and Figure 2b show the effect of addition of glucose to cells fully-adapted to growth on sorbitol. After the addition of glucose, sorbitol utilization was retarded and glucose was removed immediately at a faster rate than was sorbitol in the control, even though the culture had been previously adapted to sorbitol.

The results of a similar experiment in which young glycerol-adapted cells were used are shown in Figure 3. Six flasks were used and glucose was added to individual flasks containing glycerol at 0, 1, 2, and 3 hours. The data show that the removal of glycerol, to which the cells were adapted and which they were actively metabolizing, was severely inhibited by the introduction of glucose. The rate of glucose utilization in flasks to which it was added at zero time and at one hour (3a

Figure 2. Effect of Glucose on Utilization of Sorbitol by *E. coli* 45 Adapted to Sorbitol.

Cells were grown in 1.0 per cent sorbitol minimal medium for two days and one ml was transferred to fresh 0.2 per cent sorbitol minimal media, harvested, at 16 hours, and used to inoculate three flasks of 0.2 per cent sorbitol minimal medium and one flask of 0.2 per cent glucose minimal medium. Samples were removed for determination of substrate concentration for each flask hourly. Data for control flasks are shown in both Figure 2a and Figure 2b.

Glucose was added to a concentration of 0.2 per cent: (a) at zero time, (b) after 2 hrs. Glucose in control (○); sorbitol in control (△); glucose in mixture (●); sorbitol in mixture (▲).

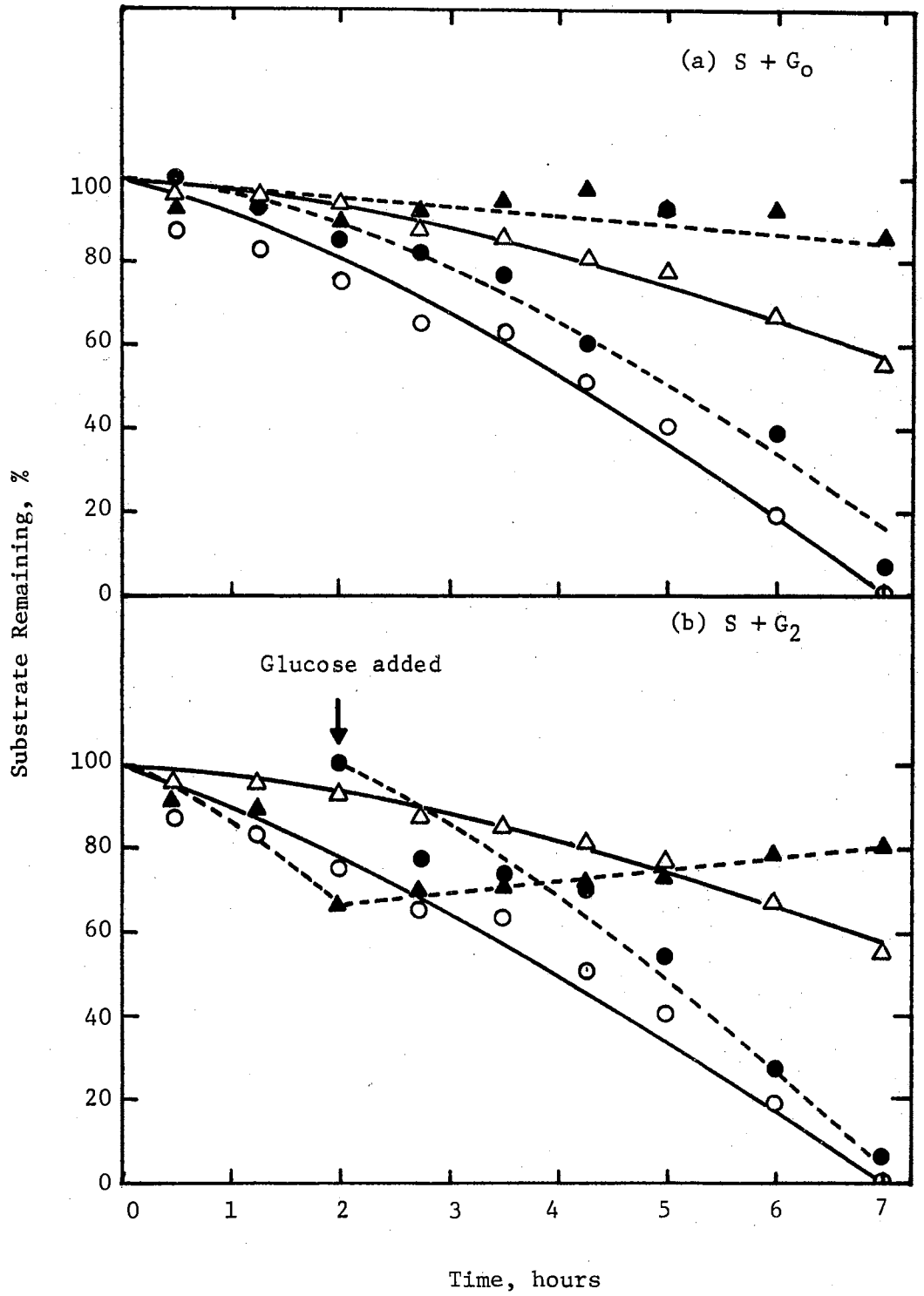
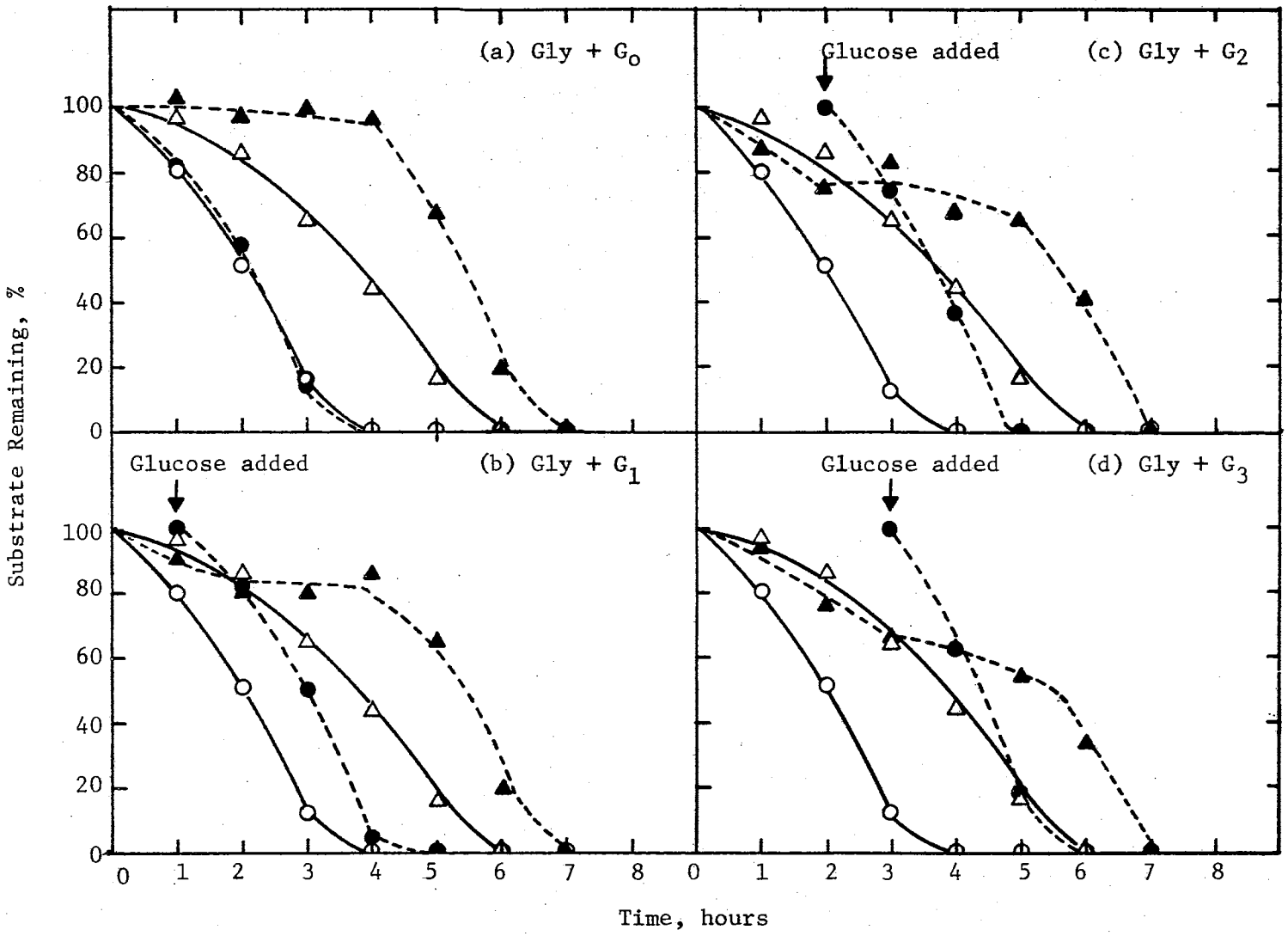

(a) S + G_o
(b) S + G_2
Glucose added
Substrate Remaining, %
Time, hours
0
20
40
60
80
100
0
1
2
3
4
5
6
7

Figure 3. Effect of Glucose on Utilization of Glycerol by E. coli 45 Adapted to Glycerol.

Cells were grown in glycerol minimal medium, harvested at 16 hours, and used to inoculate five flasks of 0.2 per cent glycerol minimal medium and one flask of 0.1 per cent glucose minimal medium. Samples were removed for determination of substrate concentration hourly for each flask. Data for control flasks are shown in all figures.

Glucose was added to a concentration of 0.1 per cent: (a) at zero time, (b) after one hour, (c) after two hours, and (d) after three hours. Glucose concentration in control flask (o), in the mixture (●); glycerol concentration in the control (△), in the mixture (▲).


(a) Gly + G_o
(b) Gly + G_1
(c) Gly + G_2
(d) Gly + G_3
Glucose added
Glucose added
Glucose added
Substrate Remaining, %
Time, hours
0
20
40
60
80
100
0 1 2 3 4 5 6 7 8

and 3b) was almost the same as in the glucose control, but when added at two and three hours, the glucose removal was faster than in the control. It is apparent, in both Figure 2 and Figure 3, that utilization of sorbitol or glycerol was promptly suspended upon introduction of glucose and was not resumed until a basal low level of glucose had been reached.

When a culture adapted to arabinose was exposed to glucose while actively metabolizing arabinose, the results shown in Figure 4 were obtained. It is seen that the rates of glucose removal in the control and in the mixtures were almost the same. Arabinose metabolism was inhibited by glucose added at zero time (Figure 4a) and at one hour (Figure 4b). Arabinose utilization continued but the rate was much slower than in the control. Arabinose removal did not recover to the same rate as in the control until all the glucose had been exhausted. When glucose was added at 2 or 3 hours (Figure 4c and Figure 4d), arabinose removal was faster than in Figure 4a or 4b, but was still slower than in the control.

Initial and final optical densities for all flasks in the experiments shown in Figures 3 and 4 are given in Table I.

Inhibition by Glucose of Utilization of Various Substrates by Young Cells of Achromobacter sp.

The effect of glucose on sorbitol metabolism by sorbitol-adapted cells of E. coli 45 has been shown in previous figures. The results obtained in glucose-sorbitol mixtures with Achromobacter sp. are quite different. Figure 5 shows these results. It is seen that glucose removal in both the control and the mixture was quite rapid. Figure 5a

Figure 4. Effect of Glucose on Utilization of Arabinose by *E. coli* 45 Adapted to Arabinose.

The experiment was performed as described for glycerol (Figure 3) except that 0.1 per cent arabinose was used instead of 0.2 per cent glycerol. Glucose was added to a concentration of 0.1 per cent: (a) at zero time, (b) after one hour, (c) after two hours, (d) after three hours. Glucose concentration in control flask (○), in the mixture (●); arabinose concentration in the control flask (△), in the mixture (▲).

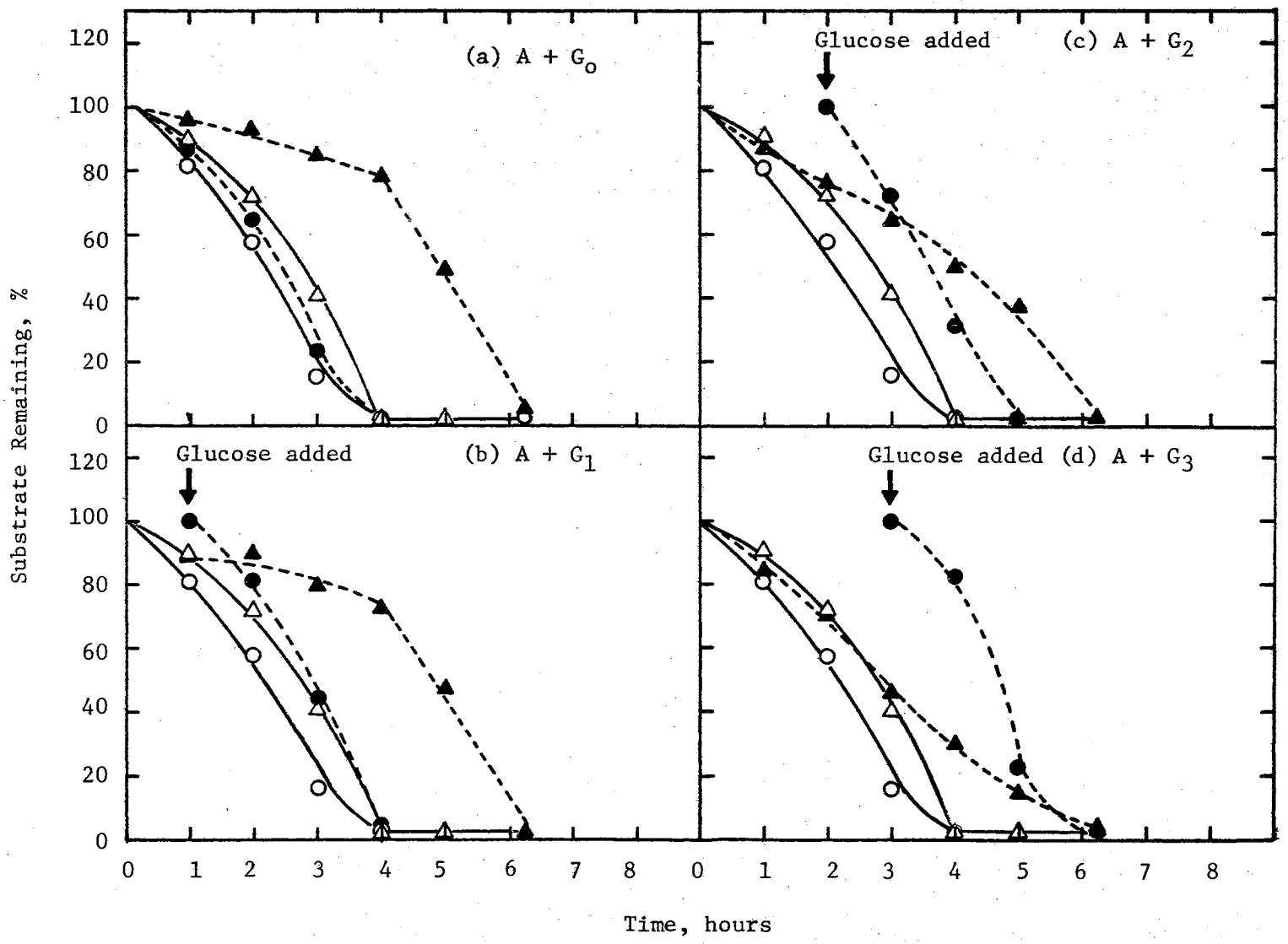

(a) A + G_o
Glucose added
(c) A + G_2
Glucose added
(b) A + G_1
Glucose added
(d) A + G_3
Substrate Remaining, %
Time, hours
0
20
40
60
80
100
120
0
1
2
3
4
5
6
7
8

TABLE I

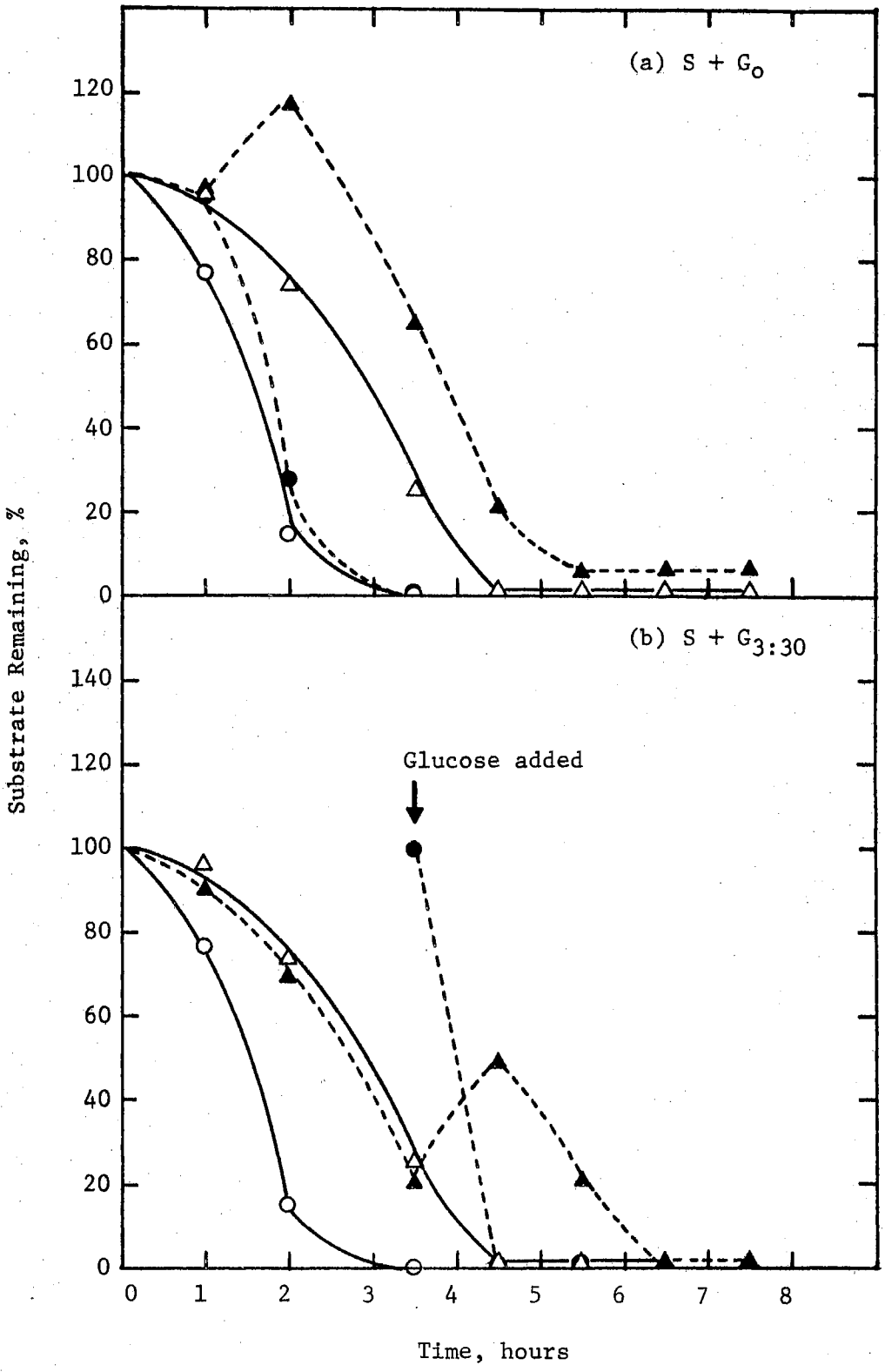
OPTICAL DENSITIES FOR ESCHERICHIA COLI 45 IN THE EXPERIMENTS ON INHIBITION BY GLUCOSE OF UTILIZATION OF VARIOUS SUGARS

Figure	Initial O. D.	Final O. D.					
		Control	Glucose Control	1st Additions	2nd Additions	3rd Additions	4th Additions
3	.138	.721	.512	.862	.894	.854	.870
4	.082	.441	.502	.643	.721	.704	.688

Experiments were carried out as described in the text. Growth readings are expressed as optical density at 540 mμ.

Figure 5. Effect of Glucose on Utilization of Sorbitol by Achromobacter sp. Adapted to Sorbitol.

Cells were grown in sorbitol medium with 0.02 per cent yeast extract, harvested after 16 hours and inoculated into three flasks of 0.1 per cent sorbitol medium and one flask of 0.1 per cent glucose medium. Glucose was added to a concentration of 0.1 per cent: (a) at zero time, (b) after three and half hours. Glucose concentration in control flask (o), in the mixture (●); sorbitol concentration in the control flask (△), in the mixture (▲).


(a) S + G_o
(b) S + $G_{3:30}$
Glucose added
Substrate Remaining, %
Time, hours
0
20
40
60
80
100
120
140
0
1
2
3
4
5
6
7
8

and Figure 5b show that sorbitol in the medium increased after addition of glucose. A repetition of this experiment with 4 flasks of glucose-sorbitol mixture gave very similar results. No controls were run for this experiment. These data are shown in Figure 6. Whether glucose was added at zero time, one hour, two hours, or three hours, sorbitol concentration in the medium increased after the glucose addition and did not decrease until after glucose had been removed.

Since the method used for determination of sorbitol is not specific for this compound, it was possible that the material apparently excreted by the cells was a metabolic intermediate, reactive to periodate, and not sorbitol itself. A number of attempts were made to identify the material by paper and thin-layer chromatography, but the amount present in the sample was insufficient for detection.

It seemed possible that the material excreted might be an intermediate derived from glucose, e.g., glycerol, which might accumulate when excess glucose was suddenly added to the medium perhaps exceeding the capacity of the available glycolytic enzymes. To test this possibility, the experiment shown in Figure 7 was carried out. Glucose was added to cells grown on glucose in the same way glucose had been added to cultures grown on sorbitol. The periodate test was used to detect excretion of periodate-positive material, correcting as usual for the glucose present, as measured by the Glucostat method. The actual amounts of glucose and of periodate-positive material calculated as glycerol are shown in Figure 8. The amounts of periodate-positive material detected were negligible.

The effect of glucose on catabolism of an amino acid is shown in Figure 9. Histidine utilization was somewhat slower after addition

Figure 6. Effect of Glucose on Utilization of Sorbitol by Achromobacter sp. Adapted to Sorbitol.

The experiment was performed as described in Figure 5. Glucose was added to a concentration of 0.1 per cent: (a) at zero time, (b) after one hour, (c) after two hours, (d) after three hours. Glucose concentration (●); sorbitol concentration (▲).

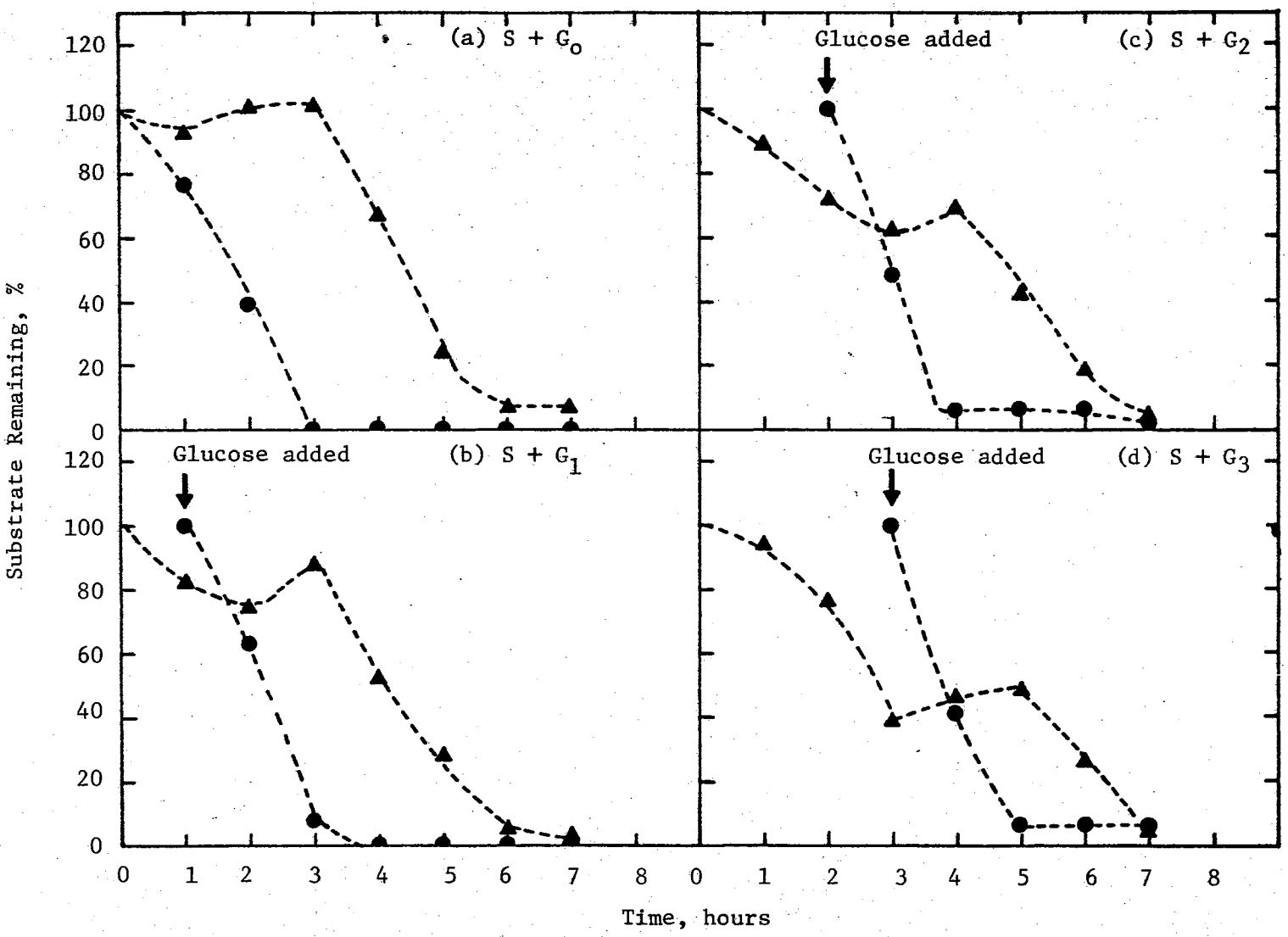

(a) S + G_o
Glucose added
(c) S + G_2
Glucose added
(b) S + G_1
Glucose added
(d) S + G_3
Substrate Remaining, %
Time, hours
0
20
40
60
80
100
120
0 1 2 3 4 5 6 7 8

Figure 7. Effect of Glucose on Utilization of Glucose by Achromobacter sp. Adapted to Glucose.

The experiment was performed as described in Figure 5 except that glucose was used instead of sorbitol and a total of six flasks was used instead of four. Glucose was added to a total concentration of 0.2 per cent: (a) at zero hour, (b) after one hour, (c) after two hours, (d) after three hours. Glucose concentration in control (o), after addition (●).

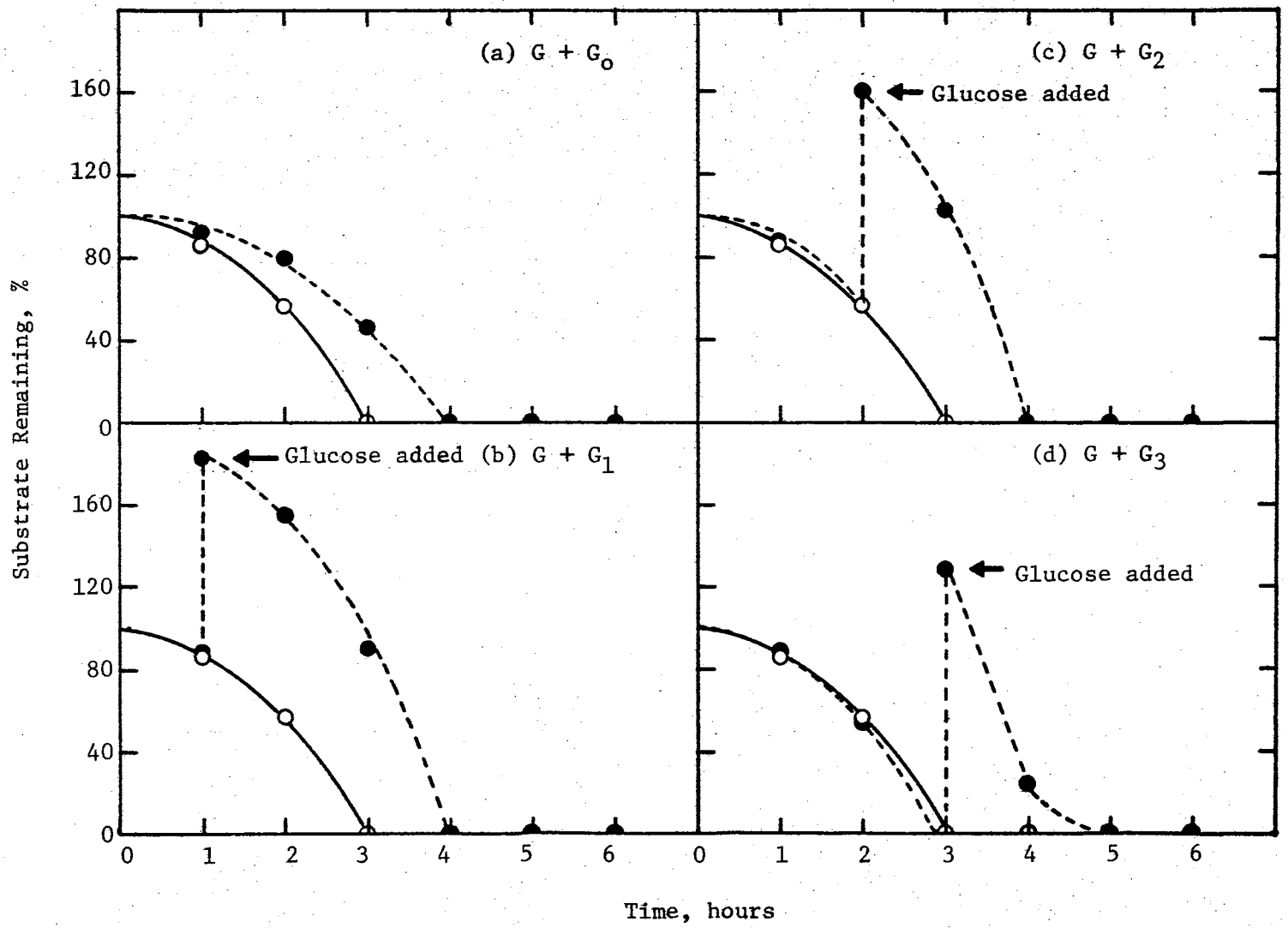

(a) G + G_o
(c) G + G_2
Glucose added
Glucose added (b) G + G_1
(d) G + G_3
Glucose added
Substrate Remaining, %
Time, hours
0
40
80
120
160
0
1
2
3
4
5
6

Figure 8. Effect of Glucose on Utilization of Glucose by Achromobacter sp. Adapted to Glucose.

Glycerol concentrations in the samples were determined by the polyol method and corrected for the amount of glucose in the sample. Glucose was added to a concentration of 0.1 per cent: (a) at zero time, (b) after one hour, (c) after two hours, (d) after three hours. Glucose concentration in the control (○), and after addition (●); glycerol concentration in the control (△) and after glucose addition (▲).

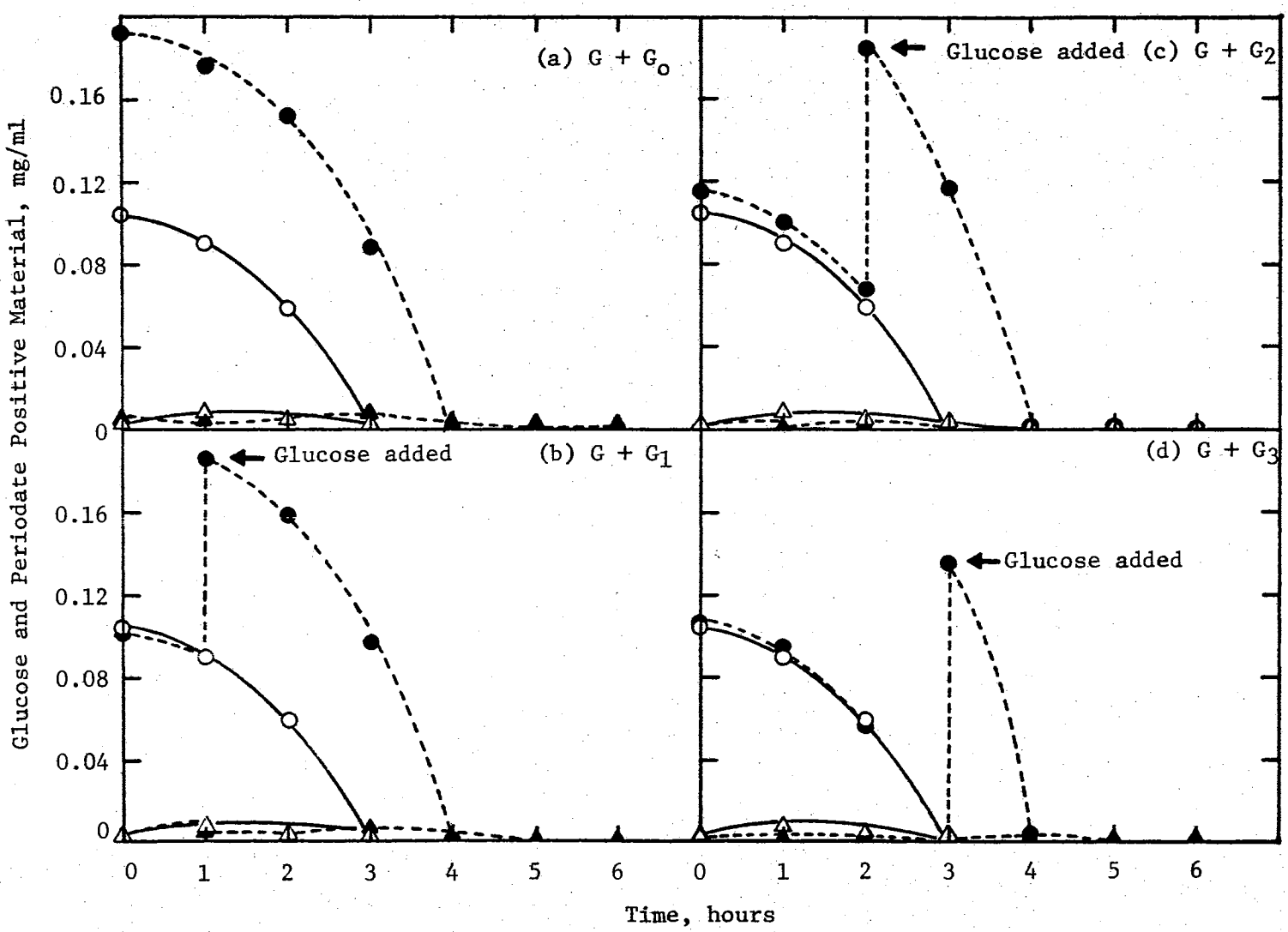
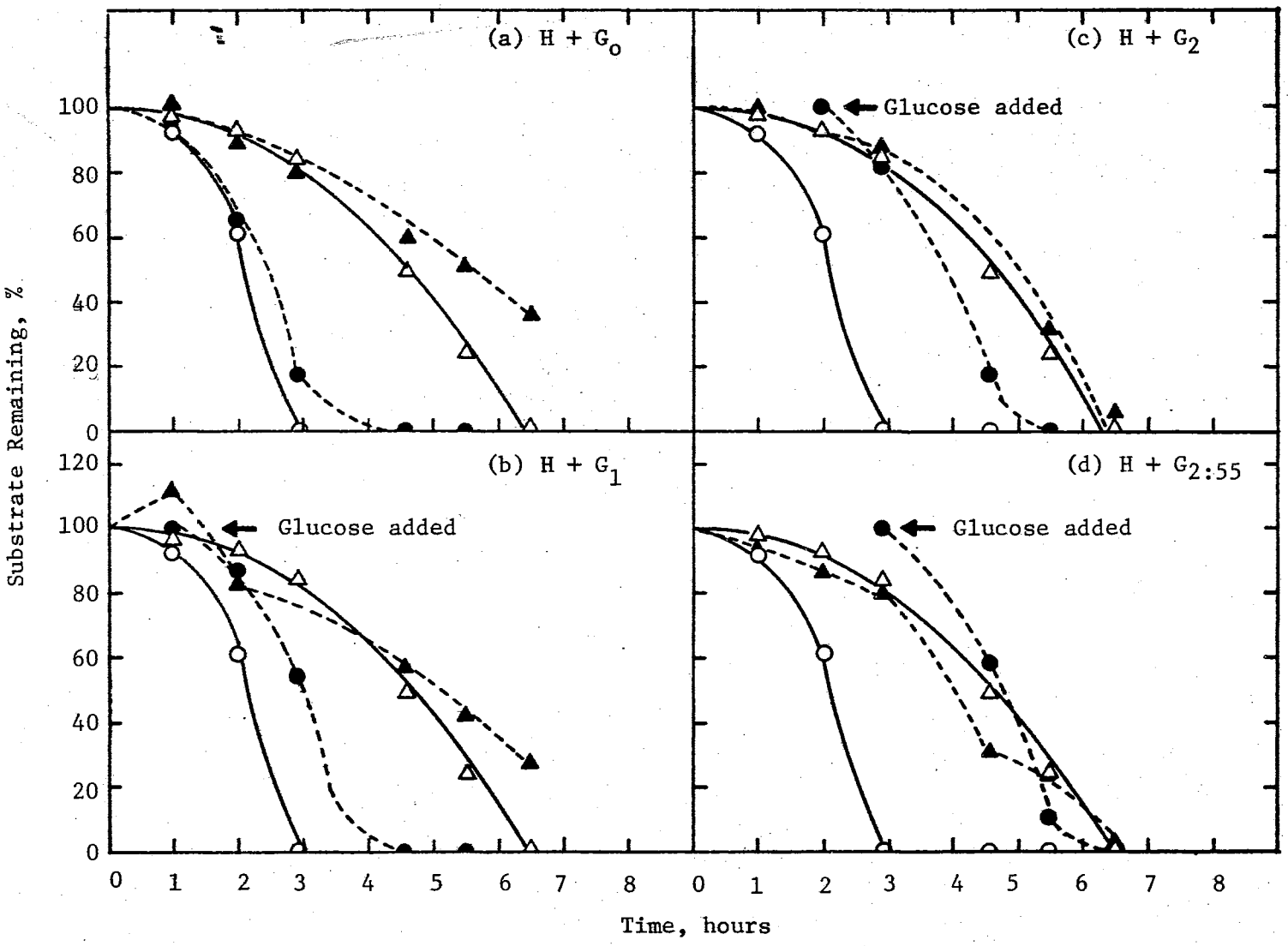

(a) G + G_o
(c) G + G_2
(b) G + G_1
(d) G + G_3
Glucose added
Glucose added
Glucose added
Glucose and Periodate Positive Material, mg/ml
0
0.04
0.08
0.12
0.16
0 1 2 3 4 5 6
Time, hours

Figure 9. Effect of Glucose on Utilization of Histidine by Achromobacter sp. Adapted to Histidine.

The experiment was performed as described in Figure 7 except using histidine instead of glucose. Glucose was added to a concentration of 0.1 per cent: (a) at zero time, (b) after one hour, (c) after two hours, (d) after 2 hours, 55 minutes. Glucose concentration in the control (○), in the mixture (●); histidine concentration in the control (△), and in the mixture (▲).


(a) H + G_o
(c) H + G_2
(b) H + G_1
(d) H + $G_{2:55}$
Glucose added
Glucose added
Glucose added
Substrate Remaining, %
Time, hours
0
20
40
60
80
100
120
0 1 2 3 4 5 6 7 8

of glucose at zero time or one hour than in the control but the overall data indicate that glucose affects histidine utilization only slightly. It is interesting to note that the addition of glucose did not cause a rapid excretion of histidine.

In studying the effect of glucose on glycerol utilization in Achromobacter sp., the results (Figure 10), in general, are similar to those for E. coli 45; i.e., the removal of glycerol, which the adapted cells were actively metabolizing, was severely inhibited upon introduction of glucose at zero time, one hour or two hours. Though glycerol removal did not completely cease after the addition of glucose at 3 hours, the rate was slower than in the control.

Results of experiments in which young arabinose- and ribose-adapted cells were used are shown in Figure 11 and Figure 12, respectively. The results in these two cases were quite different. Figure 11 shows that the rate of arabinose removal was decreased by approximately 80-90 per cent in the presence of glucose. After all the glucose had been exhausted, the rate of utilization of arabinose increased. When glucose was added to cells actively metabolizing ribose, the effect was not a complete blockage of ribose utilization (Figure 12). Removal of ribose from the substrate mixture gradually decreased in rate, as compared to the control after glucose was introduced. In general, as glucose metabolism increased, ribose metabolism was increasingly retarded.

Table II shows the optical density data for this set of experiments.

Variation in Substrate Ratios

This phase of the study was undertaken in order to determine the

Figure 10. Effect of Glucose on Utilization of Glycerol by Achromobacter sp. Adapted to Glycerol.

The experiment was performed as described in Figure 9 except using 0.2 per cent of glycerol instead of 0.1 per cent histidine. Glucose was added to a concentration of 0.1 per cent: (a) at zero time, (b) after one hour, (c) after two hours, (d) after three hours. Glucose concentration in the control (○), in the mixture (●); glycerol concentration in the control (△), in the mixture (▲).

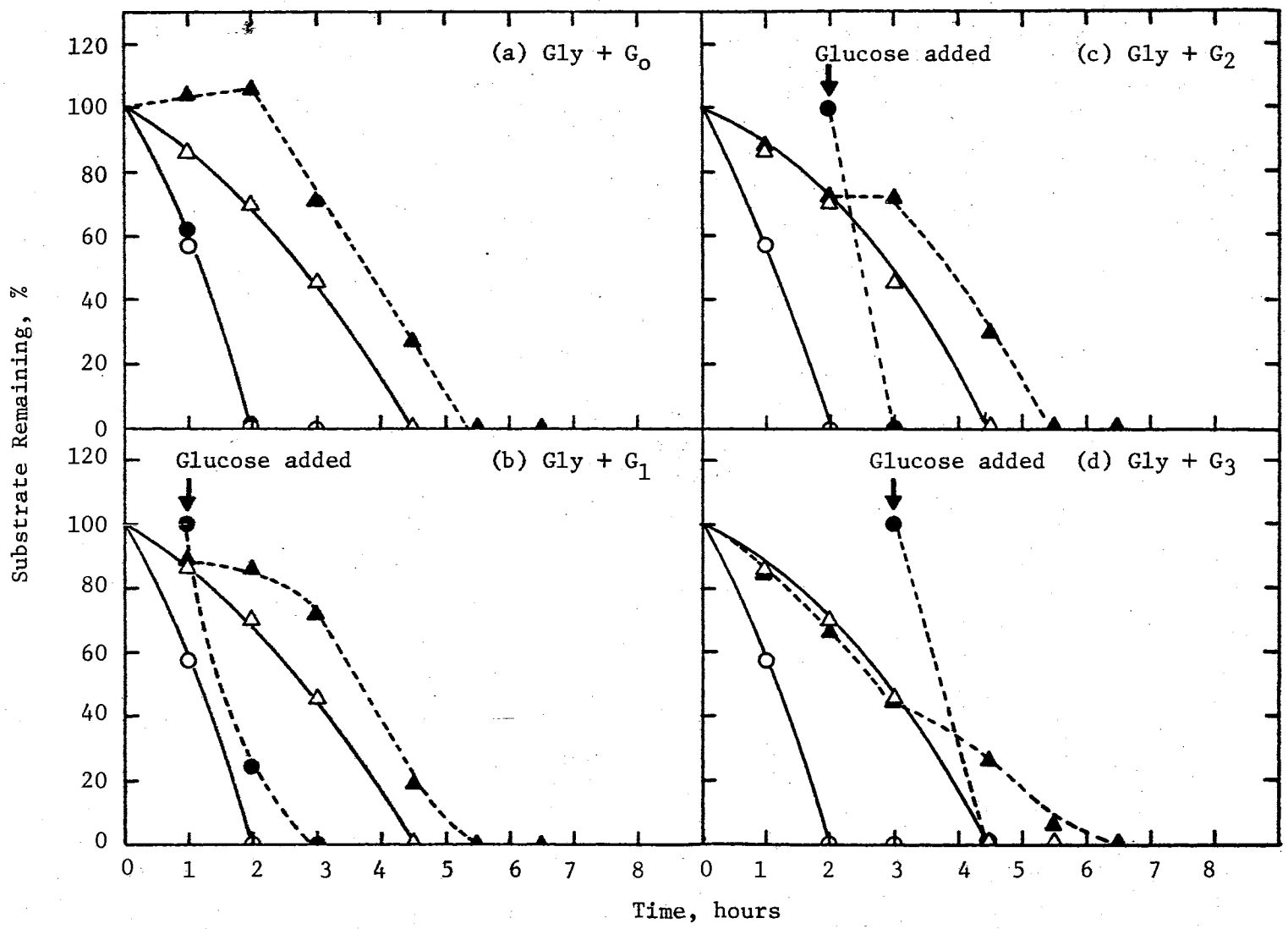

(a) Gly + G_o
(b) Gly + G_1
(c) Gly + G_2
(d) Gly + G_3
Glucose added
Glucose added
Glucose added
Glucose added
Substrate Remaining, %
Time, hours
0
20
40
60
80
100
120
0 1 2 3 4 5 6 7 8

Figure 11. Effect of Glucose on Utilization of Arabinose by Achromobacter sp. Adapted to Arabinose.

The experiment was performed as described in Figure 9 except using arabinose instead of histidine. Glucose was added to a concentration of 0.1 per cent: (a) at zero time, (b) after one hour, (c) after two hours, (d) after three hours. Glucose concentration in the control (○), in the mixture (●); arabinose concentration in the control (△), in the mixture (▲).

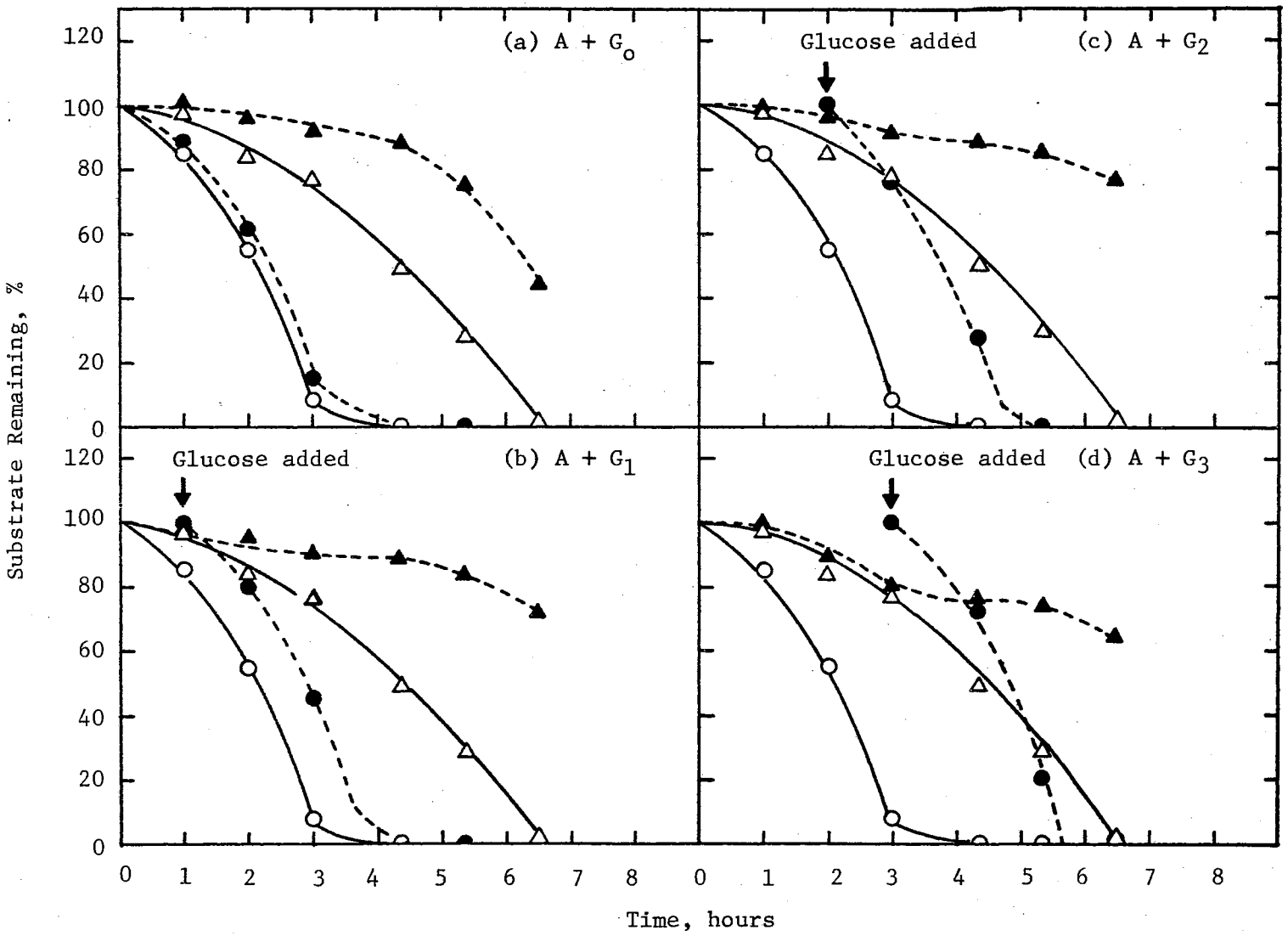

(a) A + G_o
Glucose added
(c) A + G_2
Glucose added
(b) A + G_1
Glucose added
(d) A + G_3
Substrate Remaining, %
Time, hours
0
20
40
60
80
100
120
0 1 2 3 4 5 6 7 8

Figure 12. Effect of Glucose on Utilization of Ribose by Achromobacter sp. Adapted to Ribose.

The experiment was performed as described in Figure 9 except using ribose instead of histidine. Glucose was added to a concentration of 0.1 per cent: (a) at zero time, (b) after one hour, (c) after two hours, (d) after three hours. Glucose concentration in the control (○), in the mixture (●); ribose concentration in the control (△) and in the mixture (▲).

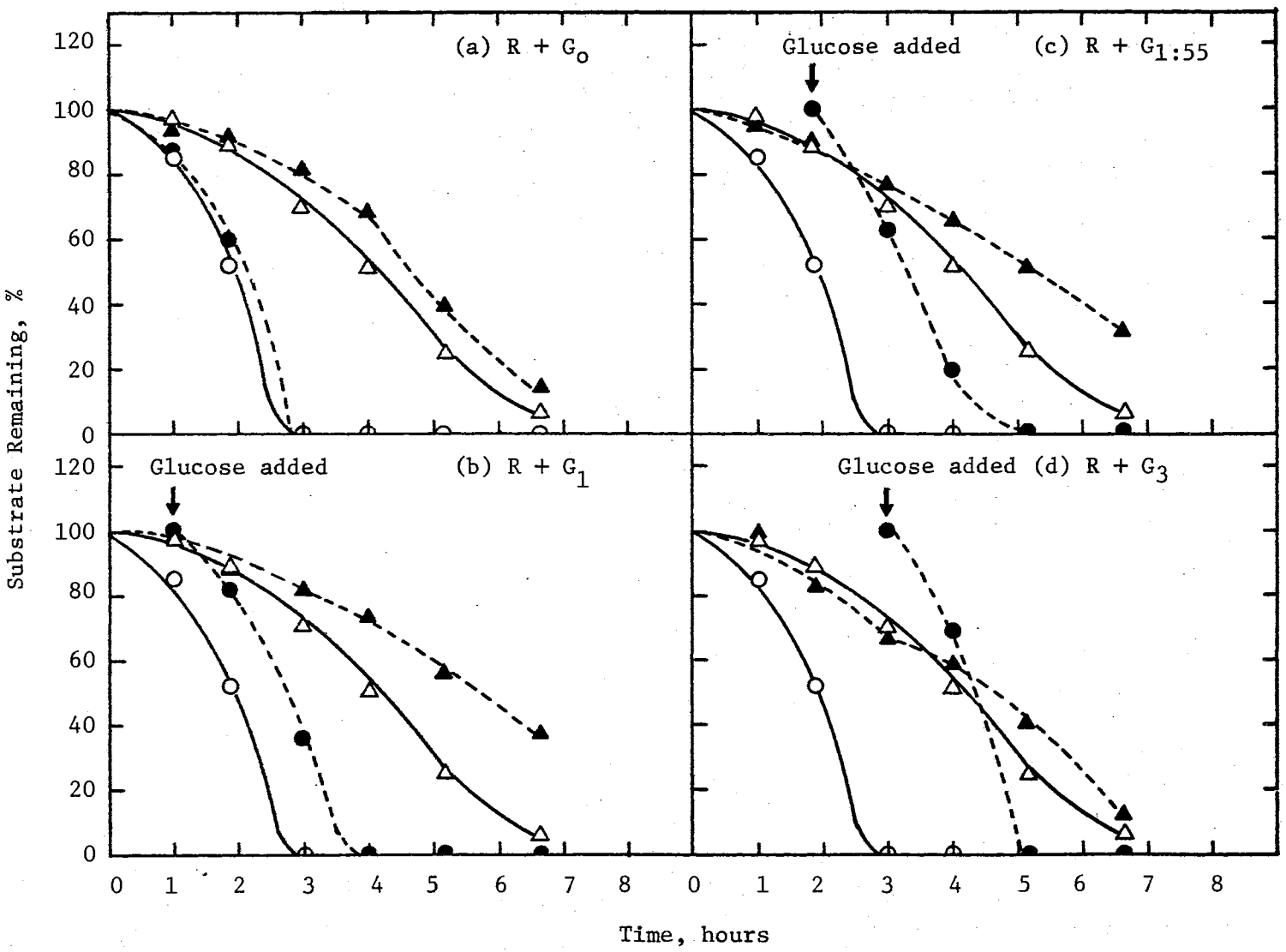

(a) R + G_o
Glucose added
(c) R + $G_{1:55}$
Glucose added
(b) R + G_1
Glucose added
(d) R + G_3
Substrate Remaining, %
Time, hours
0
20
40
60
80
100
120
0 1 2 3 4 5 6 7 8

TABLE II

OPTICAL DENSITIES FOR ACHROMOBACTER SP. IN THE EXPERIMENTS ON INHIBITION BY GLUCOSE OF UTILIZATION OF VARIOUS SUBSTRATES

Figure	Initial O. D.	Final O. D.					
		Control	Glucose Control	1st Additions	2nd Additions	3rd Additions	4th Additions
5	.103	.478	.475	.611	.678		
6	.110			.638	.683	.688	.688
7	.068		.303	.509	.530	.530	.542
9	.060	.390	.352	.392	.472	.530	.534
10	.250	.745	.611	.894	.894	.912	.912
11	.053	.344	.472	.475	.509	.520	.534
12	.078	.392	.444	.611	.573	.615	.727

Experiments were carried out as described in the text. Growth readings are expressed as optical density at 540 mμ.

interaction between various concentrations of one sugar and a fixed concentration of another sugar. When a population which had been grown on 0.1 per cent sorbitol was combined at zero time with various concentrations of glucose (0.1, .08, 0.06, 0.04 and 0.02 per cent), the effect was not a complete blockage of sorbitol utilization (Figure 13). By comparing sorbitol in the control and in the various mixtures with glucose, it is seen that for all concentrations of glucose added at zero time, sorbitol utilization was retarded until all the glucose had been exhausted. In all flasks, the rate of sorbitol metabolism was reduced for approximately two hours after introduction of glucose.

The effect of different concentrations of glucose on glycerol (Figure 14) was similar to that on sorbitol. Glycerol removal was almost completely retarded by the introduction of 0.1, 0.08, or 0.06 per cent glucose (Figures 14a to 14c). In the flask shown in Figure 14d and Figure 14e, the glucose had been completely consumed within one hour and its effect was negligible.

A similar experiment was carried out in which the concentration of glucose was kept constant at 0.1 per cent and the concentration of glycerol was varied. Utilization of glycerol by this culture, which had been acclimated to 0.2 per cent glycerol, was completely inhibited by the presence of glucose, at glycerol concentrations of 0.2, 0.16, 0.12, 0.08, or 0.04 per cent (Figure 15). Because the glycerol concentrations were different, the time required to consume the glycerol was different for each flask.

Table III shows the optical density data for this set of experiments.

Figure 13. Effect of Various Concentrations of Glucose on Utilization of Sorbitol by Achromobacter sp. Adapted to Sorbitol.

Cells were grown in sorbitol medium, harvested at 16 hours and inoculated into six flasks of 0.1 per cent sorbitol medium and one flask of 0.1 per cent glucose medium. Samples were removed for determination of substrate concentrations hourly. Glucose was added at zero time to final concentrations of: (a) 0.1 per cent, (b) 0.08 per cent, (c) 0.06 per cent, (d) 0.04 per cent and (e) 0.02 per cent. Glucose concentration in the control (○), in the mixtures (●); sorbitol concentration in the control (△), in the mixtures (▲).

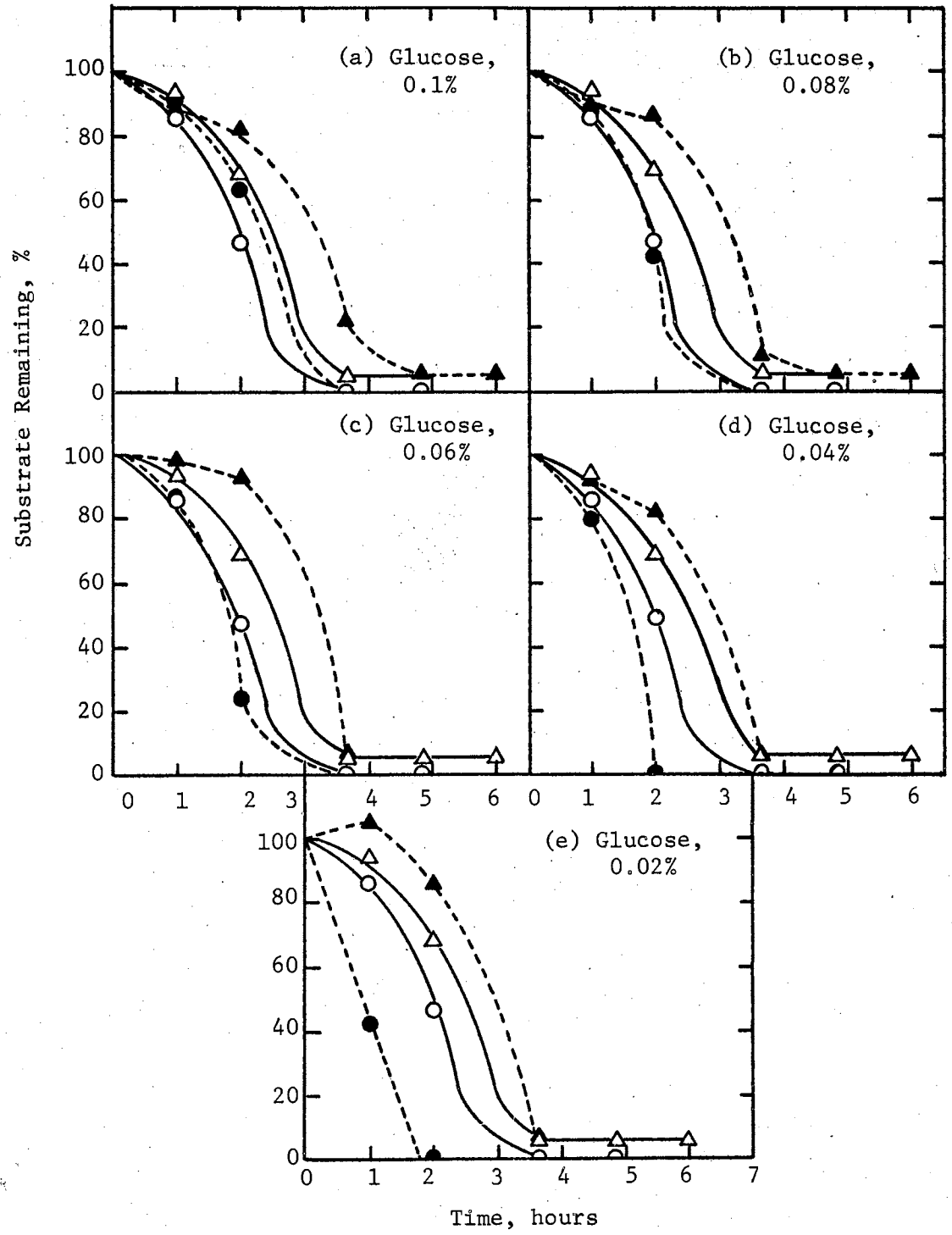

(a) Glucose, 0.1%
(b) Glucose, 0.08%
(c) Glucose, 0.06%
(d) Glucose, 0.04%
(e) Glucose, 0.02%
Substrate Remaining, %
Time, hours
0
20
40
60
80
100
0 1 2 3 4 5 6 7

Figure 14. Effect of Various Concentrations of Glucose on Utilization of Glycerol by Achromobacter sp. Adapted to Glycerol.

The experiment was performed as described in Figure 13 except using 0.2 per cent glycerol instead of 0.1 per cent sorbitol. Glucose was added at zero time to a final concentration of: (a) 0.1 per cent, (b) 0.08 per cent, (c) 0.06 per cent, (d) 0.04 per cent and (e) 0.02 per cent. Glucose concentration in the control (○), in the mixtures (●); glycerol concentration in the control (△), in the mixtures (▲).

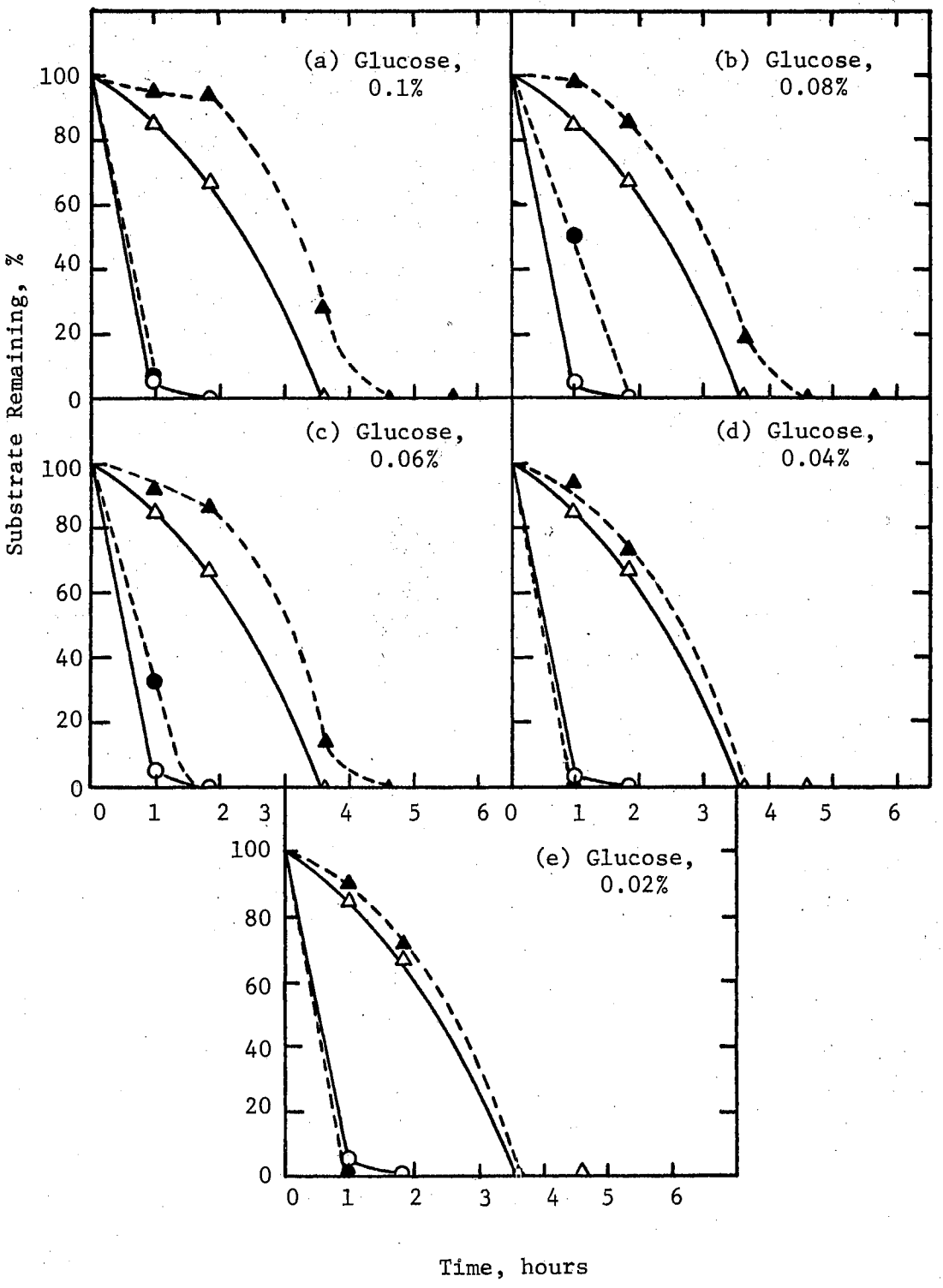

(a) Glucose, 0.1%
(b) Glucose, 0.08%
(c) Glucose, 0.06%
(d) Glucose, 0.04%
(e) Glucose, 0.02%
Substrate Remaining, %
Time, hours
0
20
40
60
80
100
0 1 2 3 4 5 6

Figure 15. Effect of Glucose on Utilization of Various Concentrations of Glycerol by Achromobacter sp. Adapted to Glycerol.

The experiment was performed as described in Figure 14 except that the concentration of glycerol was varied instead of glucose. Glucose was added at zero time to glycerol concentrations of: (a) 0.2 per cent, (b) 0.16 per cent, (c) 0.12 per cent, (d) 0.08 per cent and (e) 0.04 per cent. Glucose concentration in the control (○), in the mixtures (●); glycerol concentration in the control (△), in the mixtures (▲).

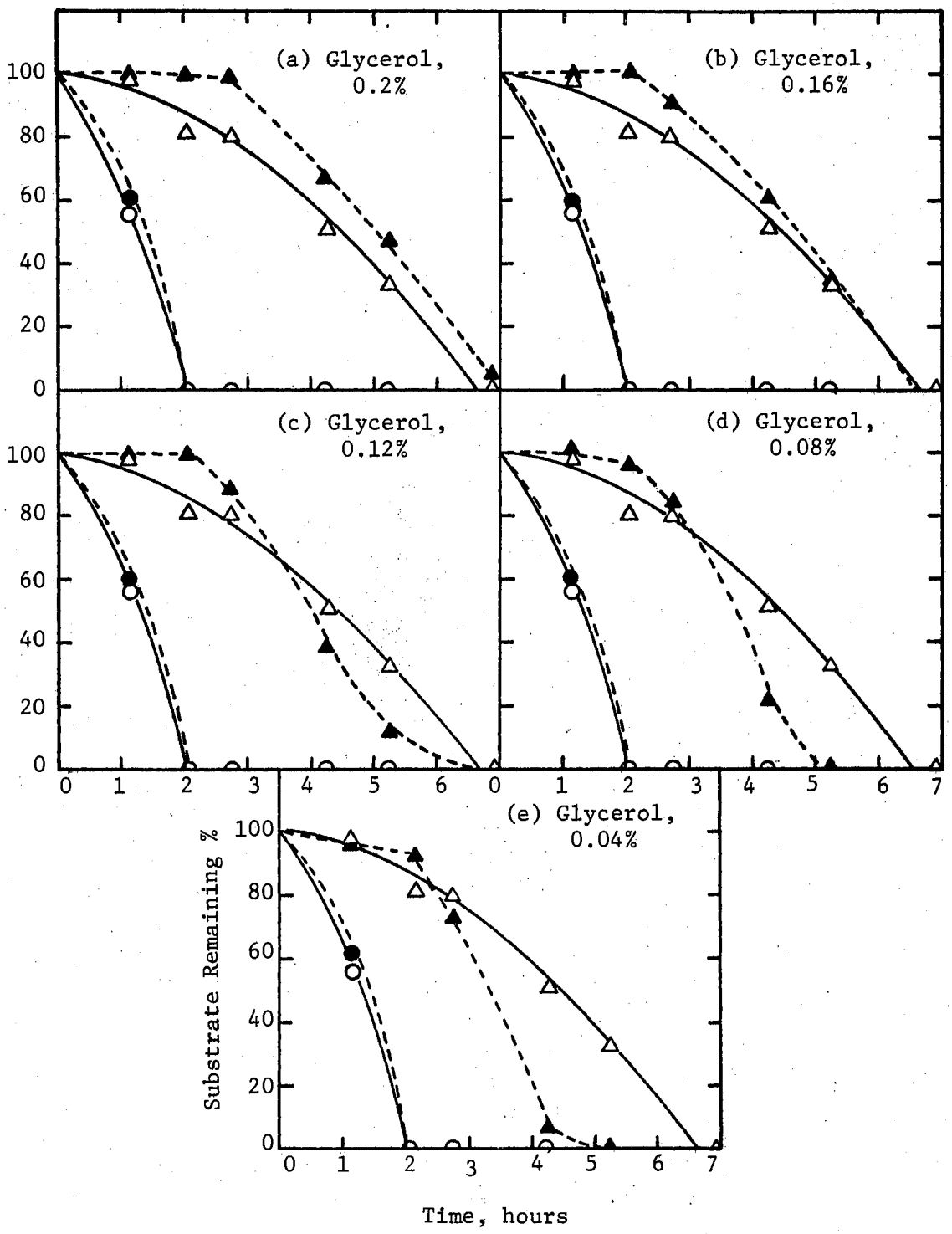

(a) Glycerol, 0.2%
(b) Glycerol, 0.16%
(c) Glycerol, 0.12%
(d) Glycerol, 0.08%
(e) Glycerol, 0.04%
Substrate Remaining %
Time, hours

TABLE III

OPTICAL DENSITIES FOR ACHROMOBACTER SP. IN THE EXPERIMENTS VARYING SUBSTRATE RATIOS

Figure	Initial O. D.	Final O. D.						
		Control	Glucose Control	1st Additions	2nd Additions	3rd Additions	4th Additions	5th Additions
13	.087	.414	.409	.573	.549	.527	.478	.462
14	.208	.668	.453	.831	.838	.810	.776	.739
15	.130	.629	.349	.733	.673	.606	.530	.472

Experiments were carried out as described in the text. Cell density is expressed as the optical density reading at 540 mμ.

Effect of Galactose on Utilization of Various Sugars by Young Cells of Escherichia coli W3110

Galactose, which is structurally related to glucose, was tested as a possibly inhibitory compound in order to determine whether the effect is limited only to glucose. The effect of galactose on removal of sorbitol is shown in Figure 16. It was observed that the rate of sorbitol utilization was slowed down slightly by introduction of galactose. However, in both the controls and in the mixtures, sorbitol was metabolized faster than was galactose and galactose was used more slowly in the presence of sorbitol than in the control.

When a population actively metabolizing ribose was exposed to galactose, there was essentially no immediate effect on ribose utilization (Figure 17). Removal of ribose in the control and in the mixture proceeded at the same rate for approximately four hours, no matter when galactose was added. By comparing ribose removal in the control and in the mixtures, it can be seen that as the rate of galactose metabolism increased, ribose removal was increasingly retarded.

Raffinose is a trisaccharide, which contains three monosaccharides, galactose, glucose and fructose. The effect of galactose on metabolism of raffinose by raffinose-grown cells is shown in Figure 18. The cells used both sugars very slowly, and it is difficult to decide whether galactose had a real effect on the metabolism of raffinose. The only case in which there was a striking difference from the control was the last addition (Figure 18d). In this instance, addition of galactose apparently caused a prolonged counterflow of raffinose. Since the test for raffinose was not specific, the material excreted could have been a

Figure 16. Effect of Galactose on Utilization of Sorbitol by E. coli W3110, Adapted to Sorbitol.

The experiment was performed as described in Figure 3, except using galactose instead of glucose, sorbitol instead of glycerol and E. coli W3110 instead of E. coli 45. Galactose was added to a final concentration of 0.1 per cent: (a) at zero time, (b) after one hour, (c) after two hours, and (d) after 2 hours, 55 minutes. Galactose concentration in the control (○), in the mixtures (●); sorbitol concentration in the control (△), in the mixtures (▲).

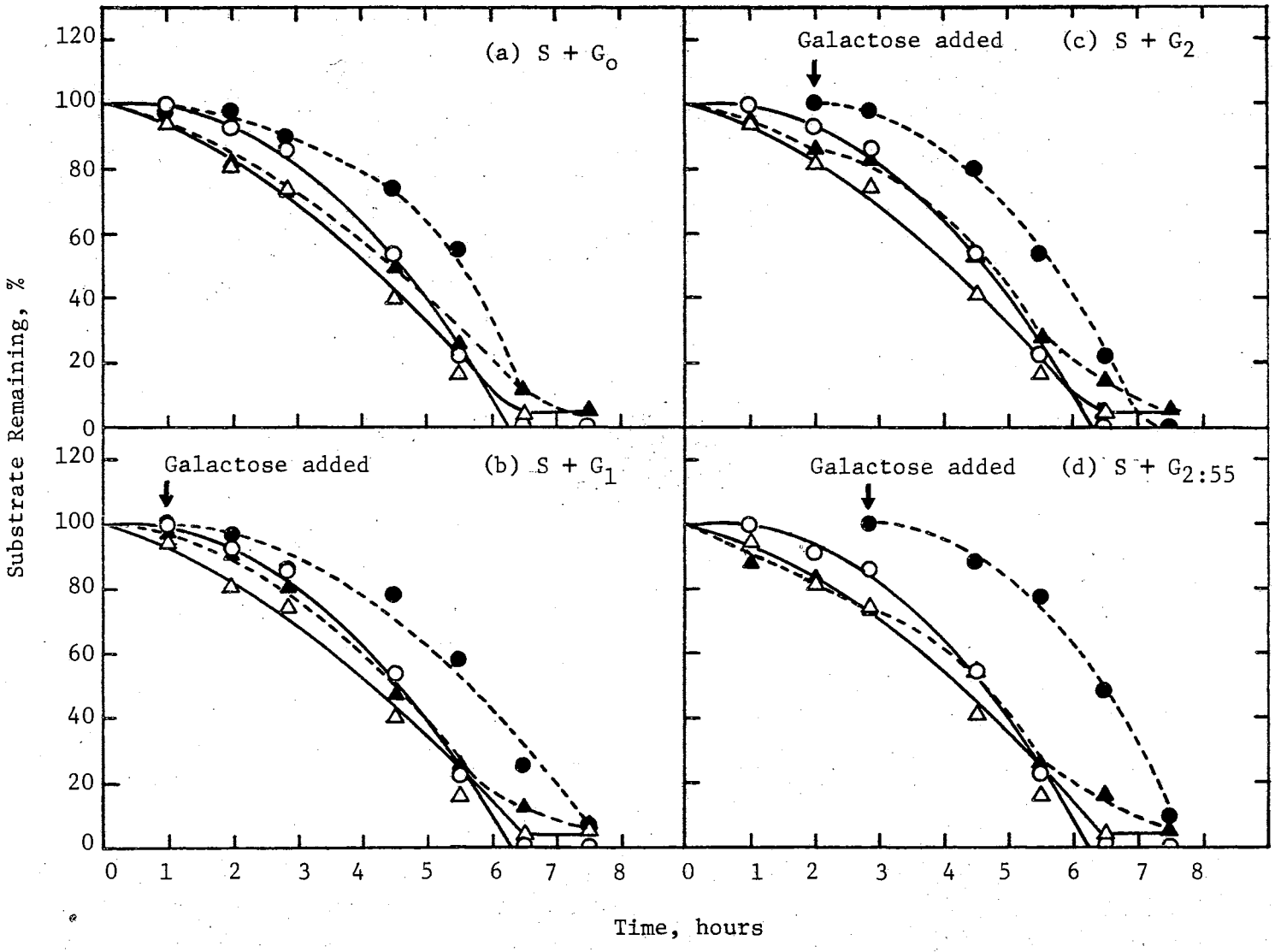

(a) S + G_o
Galactose added
(c) S + G_2
Galactose added
(b) S + G_1
Galactose added
(d) S + $G_{2:55}$
Substrate Remaining, %
Time, hours
0
20
40
60
80
100
120
0 1 2 3 4 5 6 7 8

Figure 17. Effect of Galactose on Utilization of Ribose by E. coli W3110, Adapted to Ribose.

The experiment was performed as described in Figure 16 except using ribose instead of sorbitol. Galactose was added to a final concentration of 0.1 per cent (a) at zero time, (b) after one hour, (c) after two hours, and (d) after three hours. Galactose concentration in the control (○), in the mixtures (●); ribose concentration in the control (△), in the mixtures (▲).

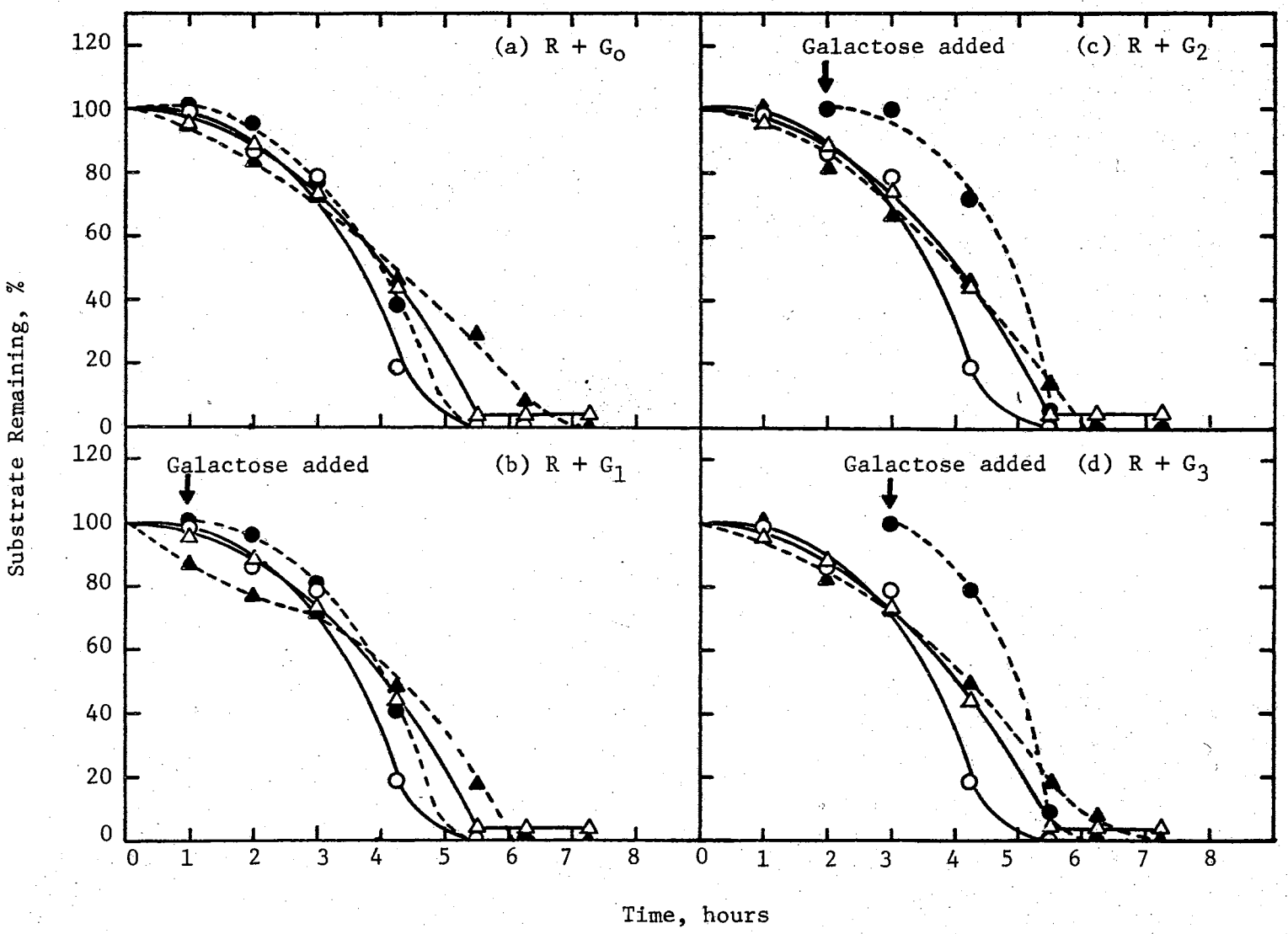
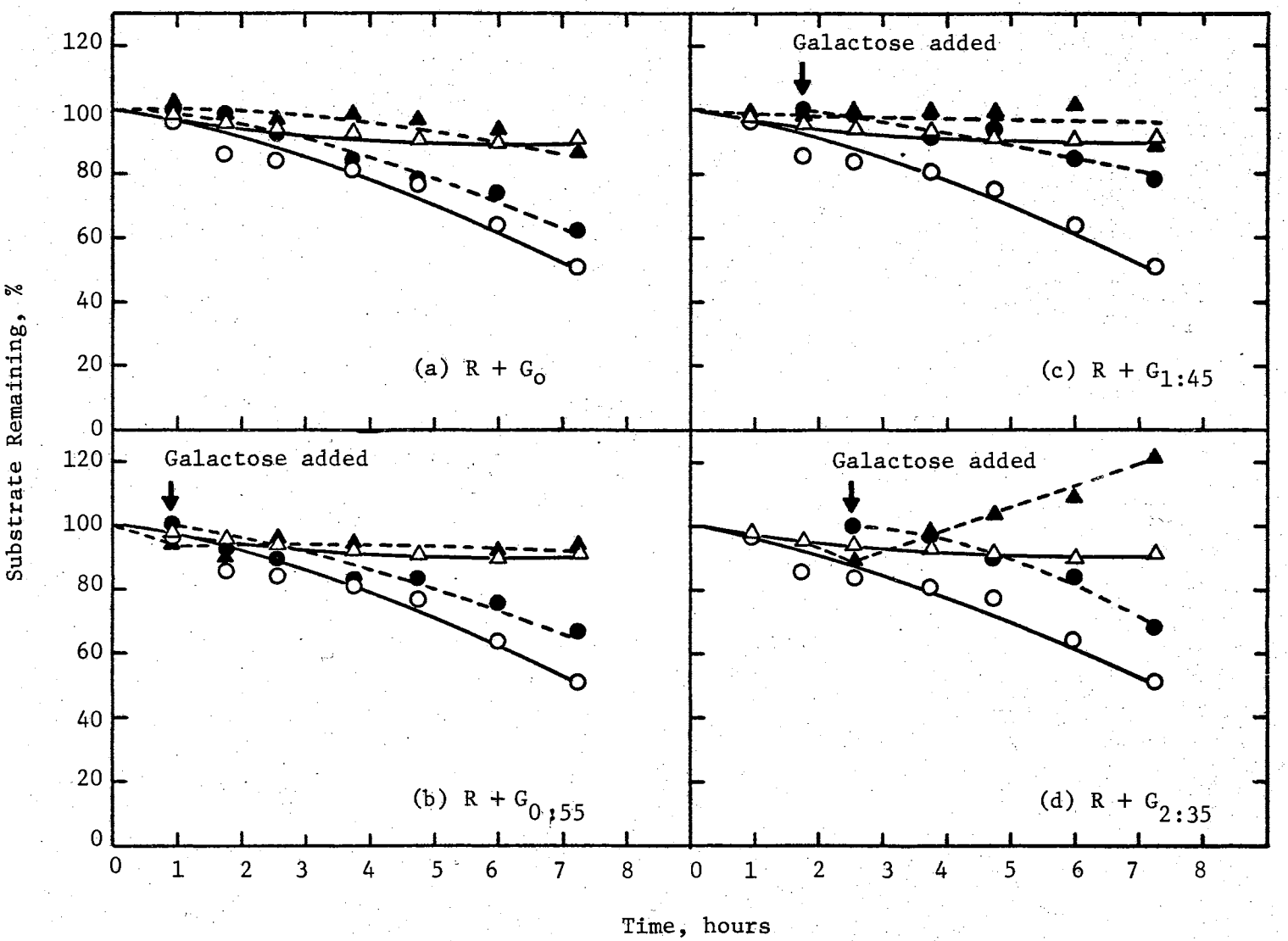

(a) R + G_o
Galactose added
(c) R + G_2
Galactose added
(b) R + G_1
Galactose added
(d) R + G_3
Substrate Remaining, %
Time, hours
0
20
40
60
80
100
120
0
1
2
3
4
5
6
7
8

Figure 18. Effect of Galactose on Utilization of Raffinose by E. coli W3110 Adapted to Raffinose.

The experiment was performed as described in Figure 16 except using raffinose instead of sorbitol. Galactose was added to a final concentration of 0.1 per cent: (a) at zero time, (b) after 55 minutes, (c) after 1 hour, 45 minutes and (d) after 2 hours 35 minutes. Galactose concentration in the control (○), in the mixtures (●); raffinose concentration in the control (△), in the mixtures (▲).


Substrate Remaining, %
120
100
80
60
40
20
0
Galactose added
(a) R + G_o
(c) R + $G_{1:45}$
(b) R + $G_{0:55}$
(d) R + $G_{2:35}$
0 1 2 3 4 5 6 7 8
Time, hours

non-reducing portion of the raffinose molecule.

Table IV shows the optical density data for this set of experiments.

Effect of Glucose on Sorbitol Metabolism by Aged Cells of Escherichia coli 45.

In all the preceding experiments, young cell population were used. It is also important to study the response of aged cells in view of the results reported for aged heterogeneous populations. The responses of the old cell populations were studied under two conditions, i.e., in cells aged without and with the addition of yeast extract.

1. Without Addition of Yeast Extract

Results of an experiment employing a six day old culture of sorbitol-grown cells are shown in Figure 19. The culture used sorbitol faster than glucose in the controls. In Figures 19a and 19b, after addition of glucose, sorbitol metabolism was inhibited just as in the young cells of E. coli 45 (Figure 2). Figure 19b also shows that sorbitol in the medium increased after addition of glucose.

The effect of glucose at daily intervals during the aging of cells is shown in Figures 20-22. The response on the first day (Figures 20a and 20b) is the same as that previously found with young cells (Figure 2). Sorbitol metabolism was completely blocked by the addition of glucose. On the second day (Figures 20c and 20d), sorbitol metabolism was again completely blocked by the introduction of glucose, but after glucose had been exhausted, sorbitol was removed at an increasing rate (Figure 20c). In both flasks (Figures 20c and 20d) after glucose was added, there was an increase in sorbitol in the medium. After the

TABLE IV

OPTICAL DENSITIES FOR ESCHERICHIA COLI W3110 IN THE EXPERIMENTS ON INHIBITION BY GALACTOSE OF UTILIZATION OF VARIOUS SUGARS

Figure	Initial O. D.	Final O. D.					
		Control	Galactose Control	1st Addition	2nd Addition	3rd Addition	4th
16	.062	.478	.465	.694	.704	.699	.704
17	.096	.545	.485	.683	.733	.796	.727
18	.020	.158	.235	.256	.256	.276	.250

Experiments were carried out as described in the text. Cell densities are expressed as optical density readings at 540 mμ.

Figure 19. Effect of Glucose on Utilization of Sorbitol by E. coli 45, Aged Cells Adapted to Sorbitol.

Cells were grown on 1.0 per cent sorbitol minimal medium overnight. On the second and third days, 2 ml were removed from the flask and replaced with 2 ml of 10 per cent sorbitol. On the fourth and fifth days, 4 ml of culture were removed from the flask and substituted with 2 ml of M-9 medium and 2 ml of 10 per cent sorbitol. On the sixth day the cells were harvested and inoculated into three flasks of 0.1 per cent sorbitol minimal medium and one flask 0.1 per cent glucose minimal medium. Samples were removed for determination of substrate concentrations hourly. Glucose was added to a final concentration of 0.1 per cent: (a) at zero time, (b) after three hours. Glucose concentration in the control (○), in the mixtures (●); sorbitol concentration in the control (△), in the mixtures (▲).

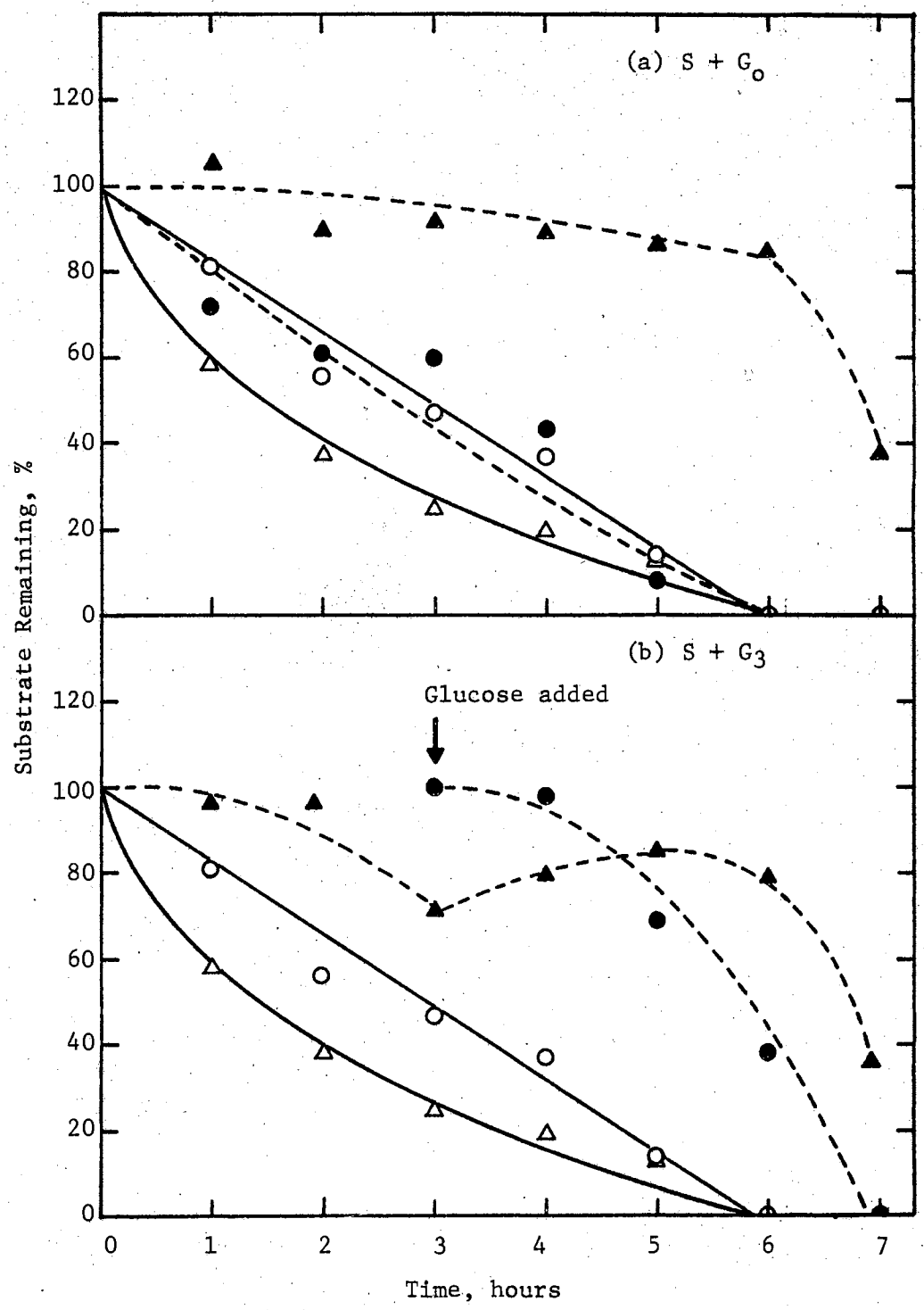

(a) S + G_o
(b) S + G_3
Glucose added
Substrate Remaining, %
Time, hours
0
20
40
60
80
100
120
1
2
3
4
5
6
7

Figure 20. Effect of Glucose on Utilization of Sorbitol by One and Two Day Old Cultures of *E. coli* 45 Adapted to Sorbitol.

Cells were grown on 0.1 per cent sorbitol minimal medium over-night in 12 flasks. Each day cells were harvested from 2 flasks and inoculated into three flasks of 0.1 per cent sorbitol minimal medium and one flask of 0.1 per cent glucose minimal medium. Samples were removed for determination of substrate concentrations hourly. The rest of the flasks were left on the shaker; 2 ml of culture were removed from each flask and replaced by 2 ml of 10 per cent sorbitol daily. Data for first and second day are shown in this figure and for third, fourth and fifth days in Figures 21 and 22. Glucose was added on the first day: (a) at zero time, (b) after 4 hours, 30 minutes; on the second day: (c) at zero time and (d) after 4 hours, 40 minutes. Glucose concentration in the control (○), in the mixtures (●); sorbitol concentration in the control (△), in the mixtures (▲).

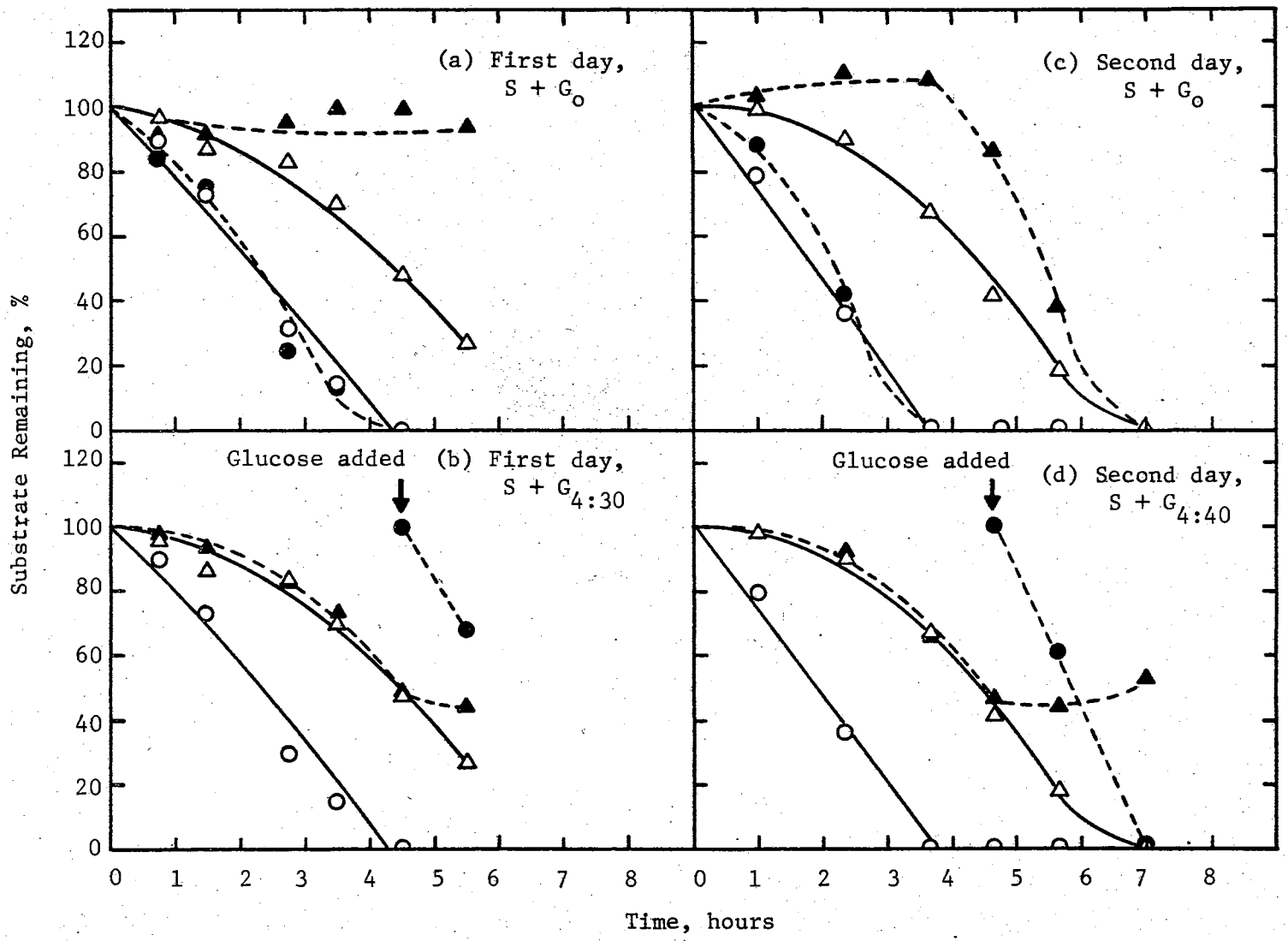

(a) First day, S + G_o
(c) Second day, S + G_o
Glucose added
(b) First day, S + $G_{4:30}$
Glucose added
(d) Second day, S + $G_{4:40}$
Substrate Remaining, %
Time, hours
0
20
40
60
80
100
120
0 1 2 3 4 5 6 7 8

Figure 21. Effect of Glucose on Utilization of Sorbitol by Three and Four Day Old Cultures of *E. coli* 45 Adapted to Sorbitol.

The experiment was performed as described in Figure 20. Glucose was added on the third day: (a) at zero time, (b) after 5 hours; on the fourth day: (c) at zero time, (d) after 4 hours, 30 minutes. Glucose concentration in the control (○), in the mixtures (●); sorbitol concentration in the control (△), in the mixtures (▲).

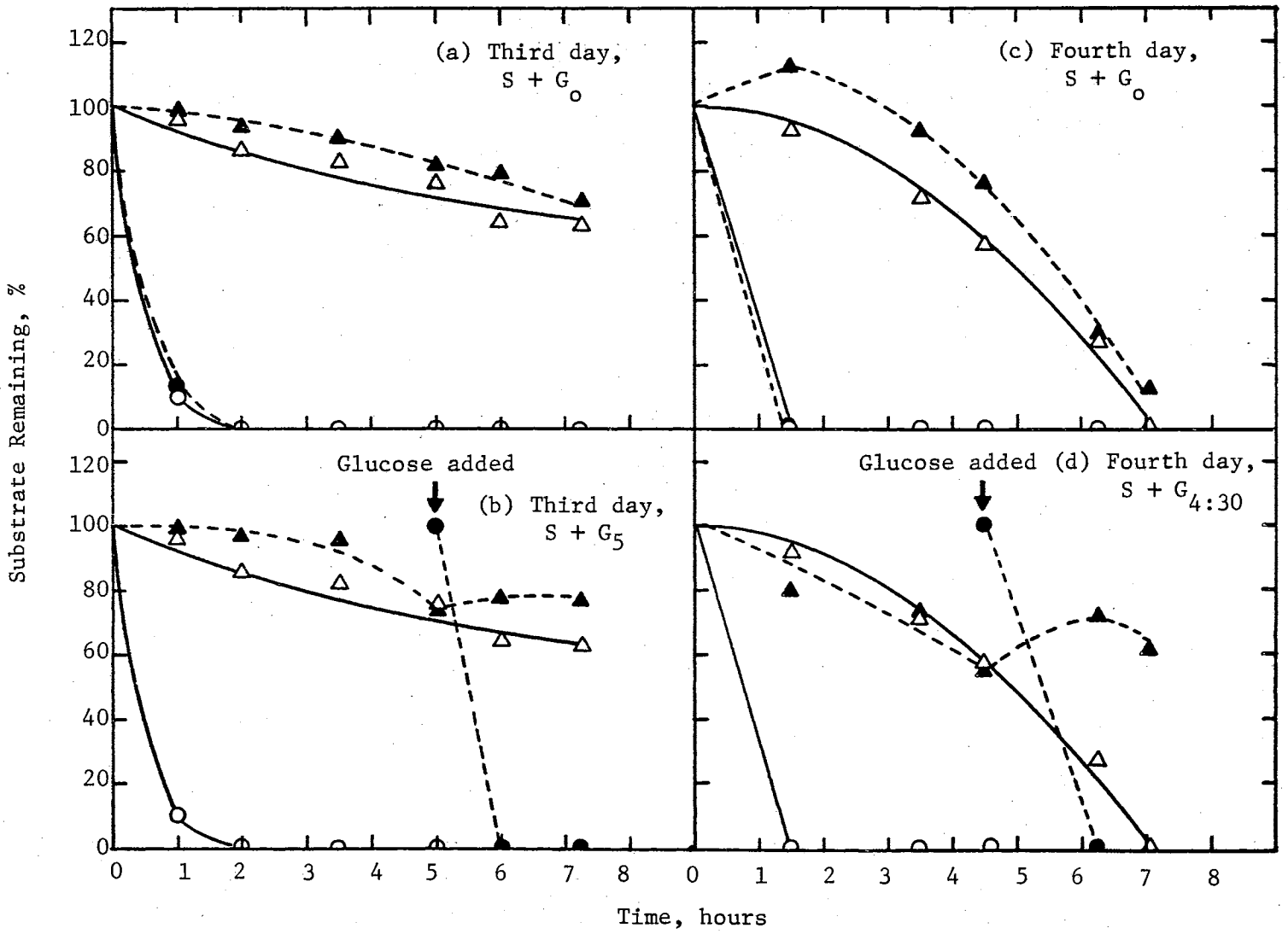
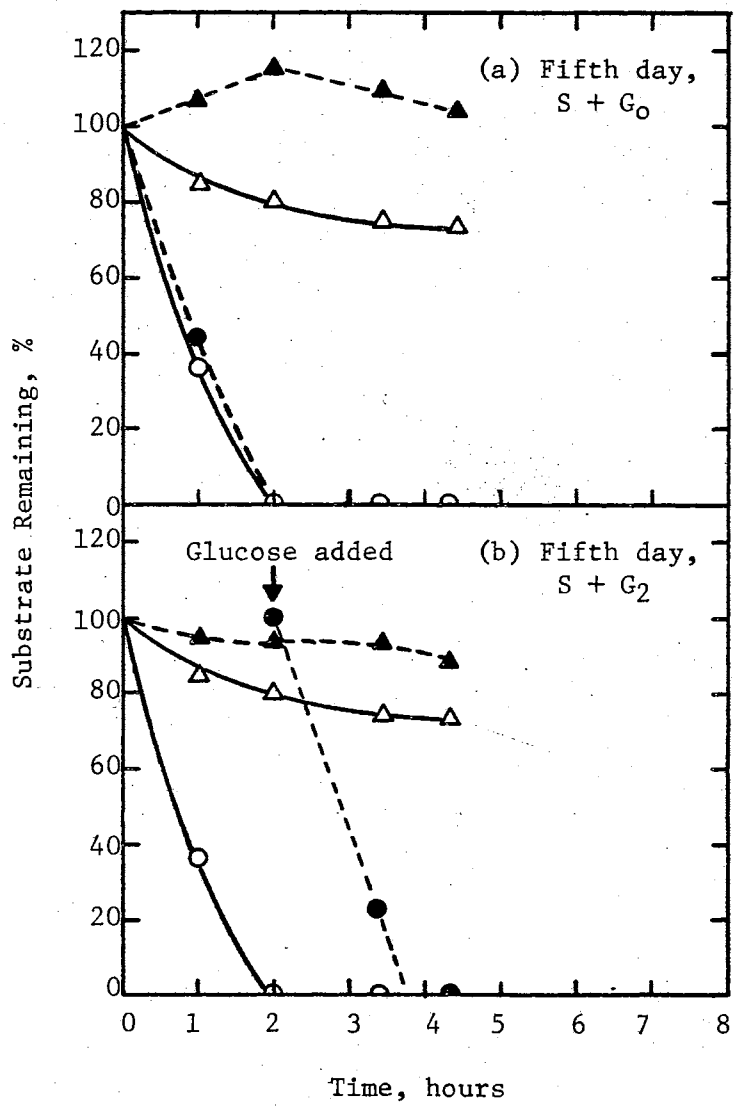

(a) Third day,
S + G$_o$
(c) Fourth day,
S + G$_o$
Glucose added
(b) Third day,
S + G$_5$
Glucose added (d) Fourth day,
S + G$_{4:30}$
Substrate Remaining, %
Time, hours
0
20
40
60
80
100
120
0 1 2 3 4 5 6 7 8

Figure 22. Effect of Glucose on Utilization of Sorbitol by a Five Day Old Culture of *E. coli* 45 Adapted to Sorbitol.

The experiment was performed as described in Figure 20. Glucose was added: (a) at zero time, (b) after 2 hours. Glucose concentration in the control (○), in the mixtures (●); sorbitol concentration in the control (△), in the mixtures (▲).


(a) Fifth day, S + G_o
(b) Fifth day, S + G_2
Glucose added
Substrate Remaining, %
Time, hours
120
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8

third day (Figures 21 and 22) sorbitol utilization was very slow, and it was inhibited by the addition of glucose. Glucose was removed very rapidly in all cases. After the addition of glucose, sorbitol concentration increased in several flasks (Figures 21b, 21c, 21d, 22a).

2. With Yeast Extract

Results of an experiment using cells aged in medium to which yeast extract was added are shown in Figures 23-24. On the first day (Figures 23a-23b) sorbitol removal was inhibited slightly by the addition of glucose at zero time and more severely by addition after one hour. By comparison with the experiments without yeast extract (Figures 20a and 20b), it is seen that both sugars were metabolized faster in the culture with yeast extract. Sorbitol removal was inhibited and sorbitol was excreted after introduction of glucose on the second day (Figures 23c and 23d), third day (Figures 24a and 24b), and fourth day (Figures 24c and 24d). Sorbitol utilization started only after glucose had been exhausted. Table V shows the optical density data for this set of experiments.

Effect of Glucose on Sorbitol Metabolism by Aged Cells of Achromobacter sp.

The effect of glucose on aged sorbitol-grown Achromobacter sp. is shown in Figures 25-27. On the first day, both glucose and sorbitol removal were slightly slower in mixtures made at zero time (Figure 25a) than in their controls. Figure 25b shows that sorbitol metabolism was retarded by the introduction of glucose. On the second day (Figures 25c and 25d), sorbitol removal was slightly inhibited by the addition

Figure 23. Effect of Glucose on Utilization of Sorbitol by One and Two Day Old Cultures of E. coli 45 Adapted to Sorbitol with Yeast Extract.

The experiment was performed as described in Figure 20 with the addition of 0.2 per cent yeast extract in the medium. Each day 2.2 ml were removed from each flask and replaced with 2 ml of 10 per cent sorbitol and 0.2 ml of 2 per cent yeast extract. Data for first and second days are shown in this figure and for third and fourth days are shown in Figure 24. Glucose was added on the first day: (a) at zero time, (b) after one hour; on the second day: (c) at zero time, and (d) after one hour. Glucose concentration in the control (○), in the mixtures (●); sorbitol concentration in the control (△), in the mixtures (▲).

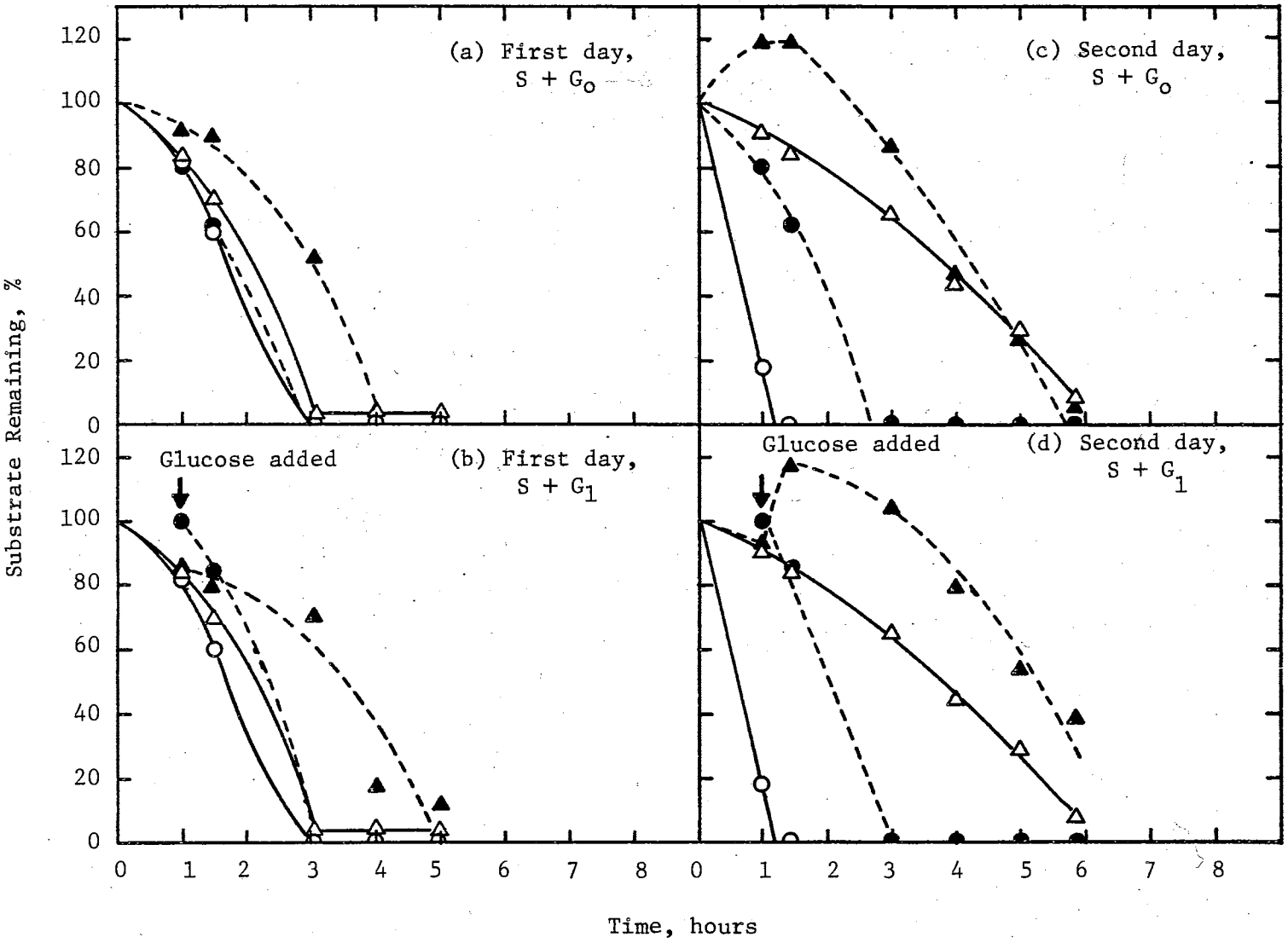

(a) First day, S + G_o
(c) Second day, S + G_o
Glucose added
(b) First day, S + G_1
Glucose added
(d) Second day, S + G_1
Substrate Remaining, %
Time, hours
0
20
40
60
80
100
120
0 1 2 3 4 5 6 7 8

Figure 24. Effect of Glucose on Utilization of Sorbitol by Three and Four Day Old Cultures of E. coli 45 Adapted to Sorbitol with Yeast Extract.

The experiment was performed as described in Figure 23. Glucose was added on the third day: (a) at zero time, (b) after 3 hours; on the fourth day: (c) at zero time, (d) after 2 hours, 55 minutes. Glucose concentration in the control (○), in the mixtures (●); sorbitol concentration in the control (△), in the mixtures (▲).

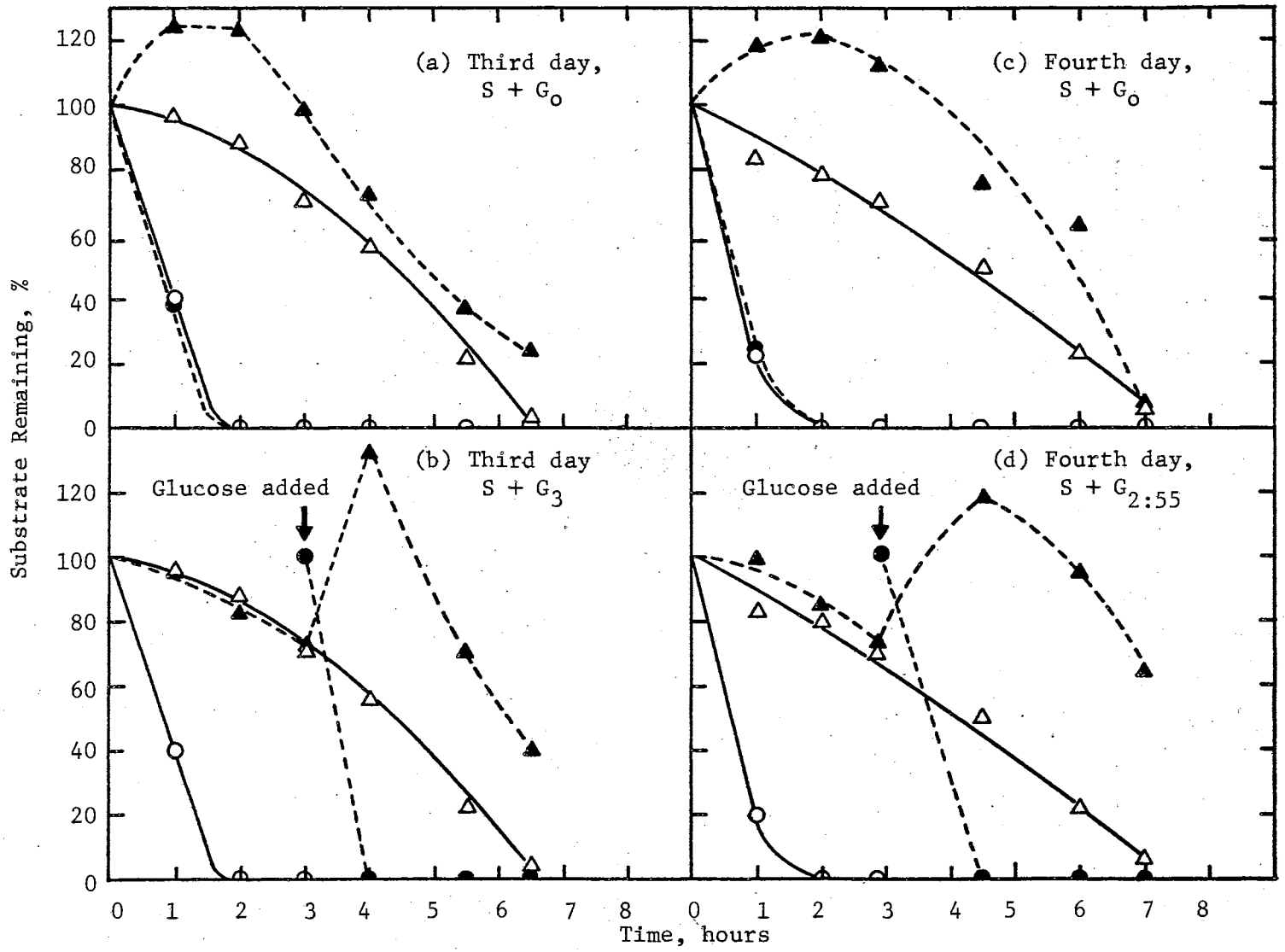

(a) Third day,
S + G_o
(c) Fourth day,
S + G_o
(b) Third day
S + G_3
Glucose added
(d) Fourth day,
S + $G_{2:55}$
Glucose added
Substrate Remaining, %
Time, hours
0
20
40
60
80
100
120
0 1 2 3 4 5 6 7 8

TABLE V

OPTICAL DENSITIES FOR ESCHERICHIA COLI 45 IN THE EXPERIMENTS ON EFFECT OF GLUCOSE ON SORBITOL METABOLISM BY AGED CELLS

Figure	Aged (days)	Initial O. D.	Final O. D.			
			Control	Glucose Control	1st Additions	2nd
20	1	.188	.629	.498	.620	.854
20	2	.201	.565	.581	.831	.824
21	3	.470	.658	.678	.751	.846
21	4	.597	.803	.745	.939	.949
22	5	.512	.789	.757	.886	.921
23	1	.095	.530	.523	.668	.716
23	2	.420	.653	.649	.846	.817
24	3	.455	.694	.721	.831	.838
24	4	.389	.782	.782	.878	.903

Experiments were carried out as described in the text. Cell densities are expressed as optical density readings at 540 mμ.

Figure 25. Effect of Glucose on Utilization of Sorbitol by One and Two Day Old Cultures of Achromobacter sp. Adapted to Sorbitol.

The experiment was performed as described in Figure 23 except using Achromobacter sp. instead of E. coli 45. Data for first and second days are shown in this figure and for third, fourth and fifth days in Figures 26 and 27. Glucose was added on the first day: (a) at zero time, (b) after two hours; on the second day: (c) at zero time, (d) after three hours. Glucose concentration in the control (○), in the mixtures (●); sorbitol concentration in the control (△), in the mixtures (▲).

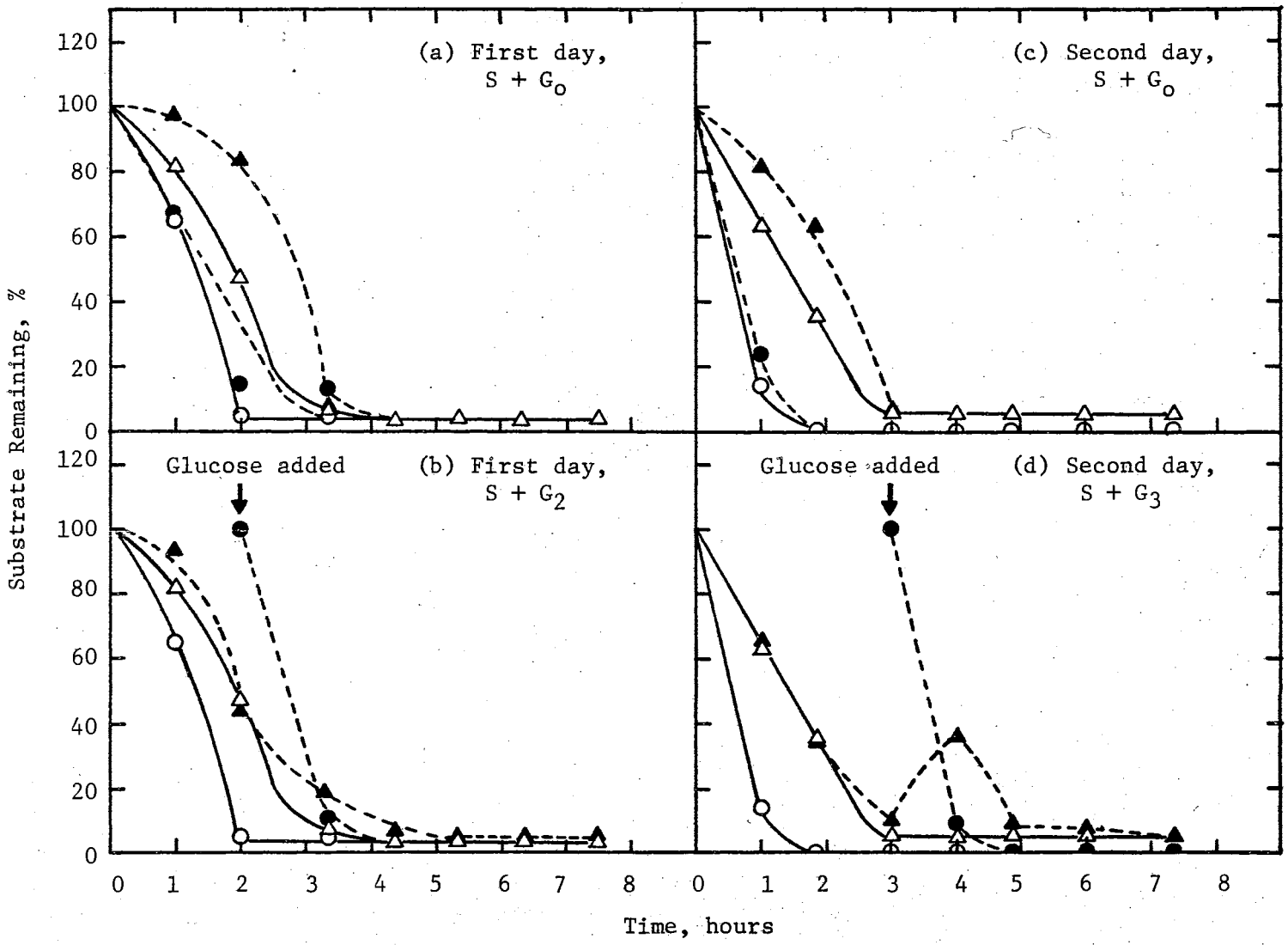

(a) First day, S + G_o
(c) Second day, S + G_o
Glucose added
(b) First day, S + G_2
Glucose added
(d) Second day, S + G_3
Substrate Remaining, %
Time, hours
0
20
40
60
80
100
120
0 1 2 3 4 5 6 7 8

Figure 26. Effect of Glucose on Utilization of Sorbitol by Three and Four Day Old Cultures of Achromobacter sp. Adapted to Sorbitol.

The experiment was performed as described in Figure 25. Glucose was added on the third day: (a) at zero time, (b) after 3 hours, 50 minutes; on the fourth day: (c) at zero time, and (d) after 3 hours, 40 minutes. Glucose concentration in the control (○), in the mixtures (●); sorbitol concentration in the control (△), in the mixtures (▲).

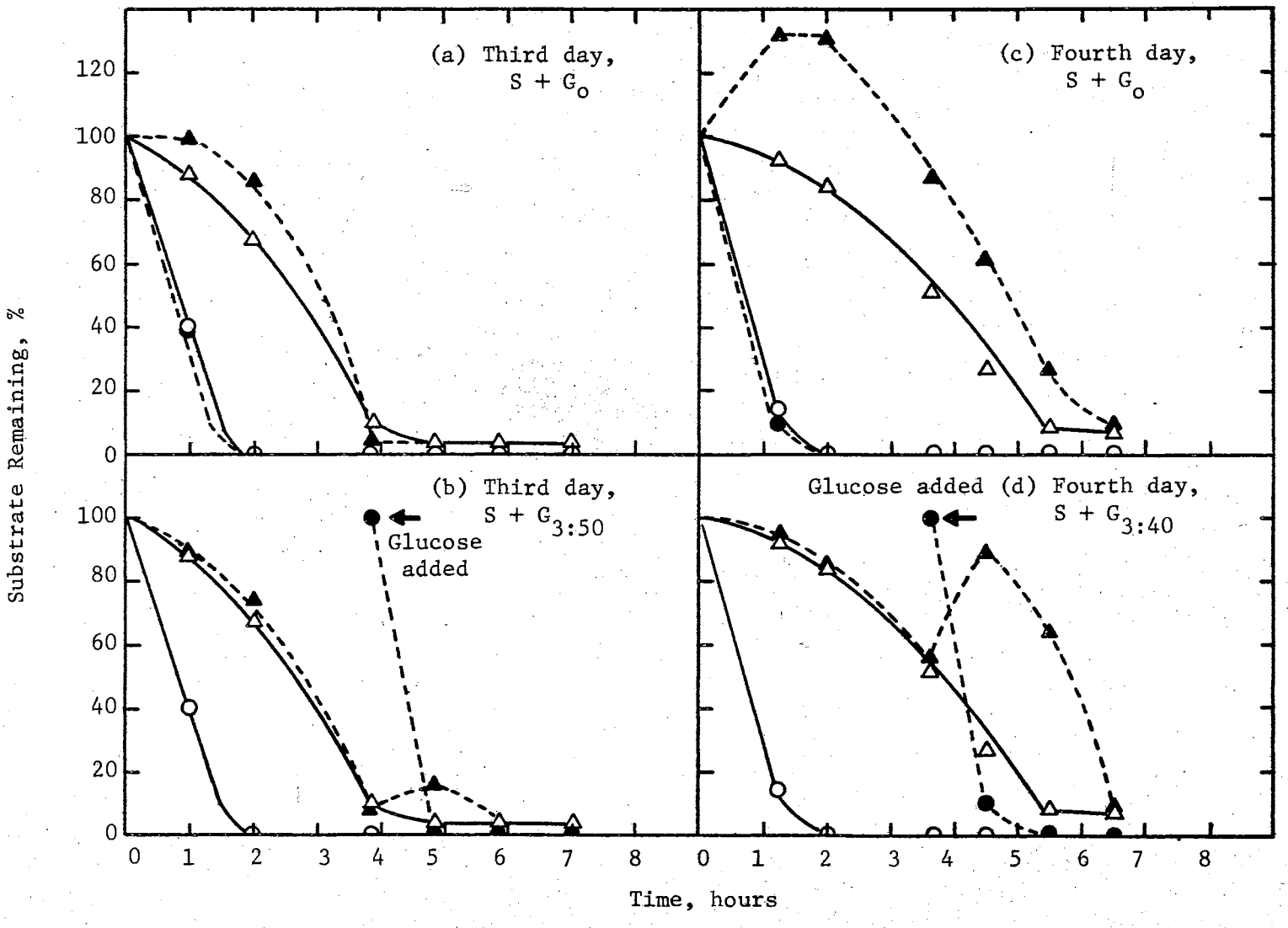
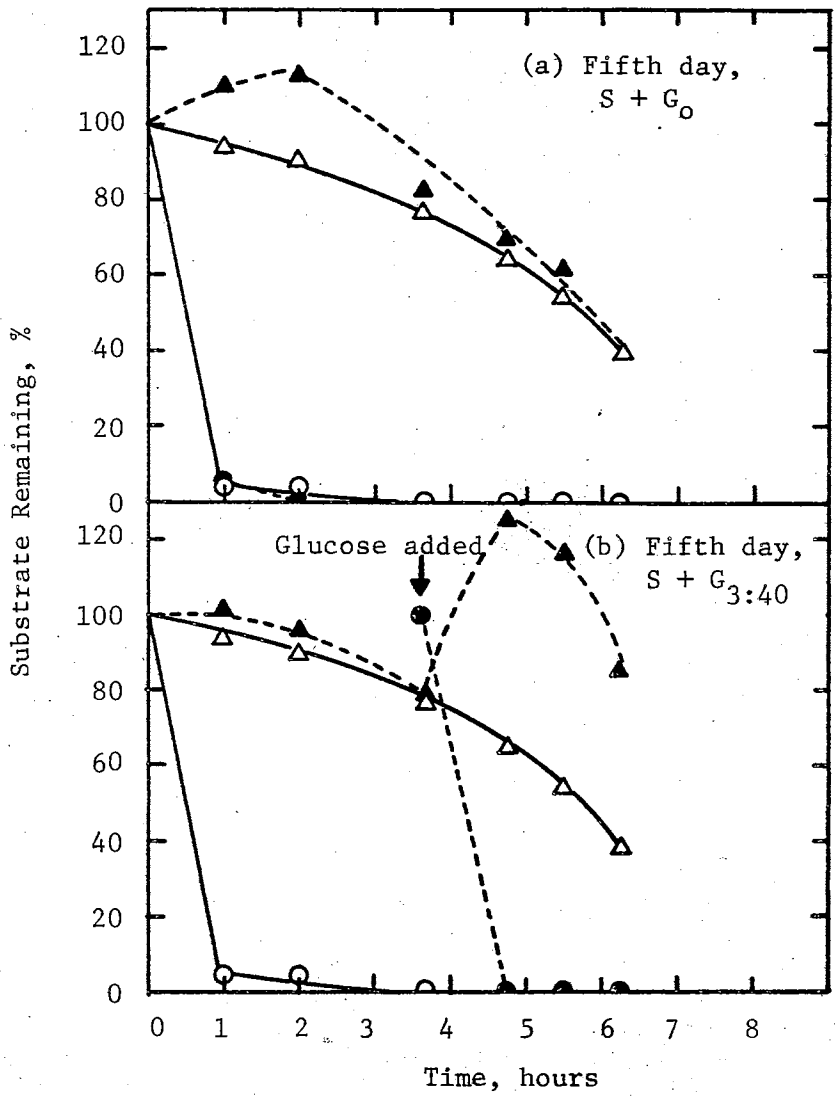

(a) Third day,
S + G$_o$
(c) Fourth day,
S + G$_o$
(b) Third day,
S + G$_{3:50}$
Glucose
added
Glucose added (d) Fourth day,
S + G$_{3:40}$
Substrate Remaining, %
Time, hours
0
20
40
60
80
100
120
0 1 2 3 4 5 6 7 8

Figure 27. Effect of Glucose on Utilization of Sorbitol by a Five Day Old Culture of Achromobacter sp. Adapted to Sorbitol.

The experiment was performed as described in Figure 25. Glucose was added: (a) at zero time, (b) after 3 hours, 40 minutes. Glucose concentration in the control (○), in the mixtures (●); sorbitol concentration in the control (△), in the mixtures (▲).


(a) Fifth day,
S + G$_o$
Glucose added
(b) Fifth day,
S + G$_{3:40}$
Substrate Remaining, %
Time, hours
0
20
40
60
80
100
120
0 1 2 3 4 5 6 7 8

of glucose at zero time (Figure 25c) and sorbitol was excreted after introduction of glucose at 3 hours (Figure 25d). The results on the third day (Figures 26a and 26b) were very similar to those on the second day. After addition of glucose on the fourth day (Figures 26c and 26d) and fifth day (Figures 27a and 27b), a considerable amount of sorbitol was excreted; after glucose had been exhausted, sorbitol utilization began.

As in the case of E. coli, the rate of sorbitol utilization decreased progressively as the cells aged, even though sorbitol was added to the culture each day. However, the ability to metabolize glucose rapidly was retained.

Table VI shows the optical density data for this set of experiments.

Continuous Flow System

Escherichia coli 45 was grown overnight with aeration at 37°C on the shaker in M-9 medium which contained 0.2 per cent sorbitol. Cells were transferred to the chemostat, which was filled to the overflow line with the same medium. Aeration was started and the temperature was set at 37°C. After three hours the pump was turned on, set at 38 per cent of capacity and optical density readings were made every hour using the effluent. After 24 hours operation, the water bath burned out and operation was continued at room temperature (24°C). Optical density readings showed that the cells were being washed out at this temperature and dilution rate. Therefore, the pumping rate was changed to 19 per cent of capacity. After 48 hours, the cells still had not grown up. The pump was turned off to allow the cells to grow. At 57 hours pumping was started again at 4 per cent of capacity. This rate was maintained for 3 days to ensure that the population had reached equilibrium. The

TABLE VI

OPTICAL DENSITIES FOR ACHROMOBACTER SP. IN THE EXPERIMENTS ON EFFECT OF GLUCOSE ON SORBITOL METABOLISM BY AGED CELLS

Figure	Aged (days)	Initial O. D.	Final O. D.			
			Control	Glucose Control	1st Additions	2nd Additions
25	1	.155	.505	.498	.678	.716
25	2	.512	.745	.716	.838	.831
26	3	.527	.688	.688	.810	.838
26	4	.457	.688	.716	.810	.854
27	5	.549	.727	.789	.831	.789

Experiments were carried out as described in the text. Cell densities are expressed as optical density readings at 540 mμ.

Figure 28. Effect of Glucose on Utilization of Sorbitol by E. coli 45 Adapted to Sorbitol in Continuous Flow.

The experiment was performed as described in the text. Glucose concentration in the control (○), in the mixture (●); sorbitol concentration in the control (△), in the mixture (▲).

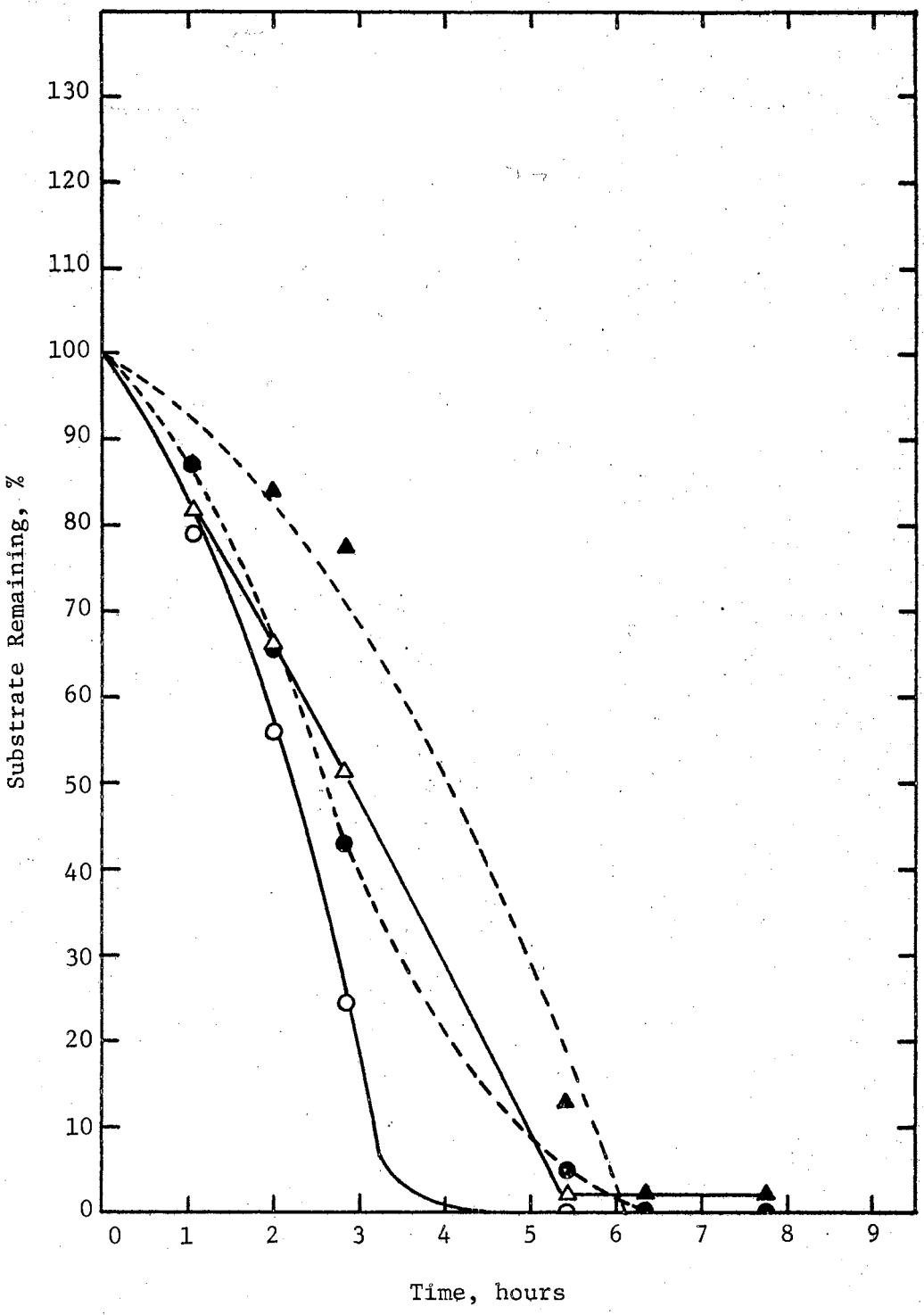
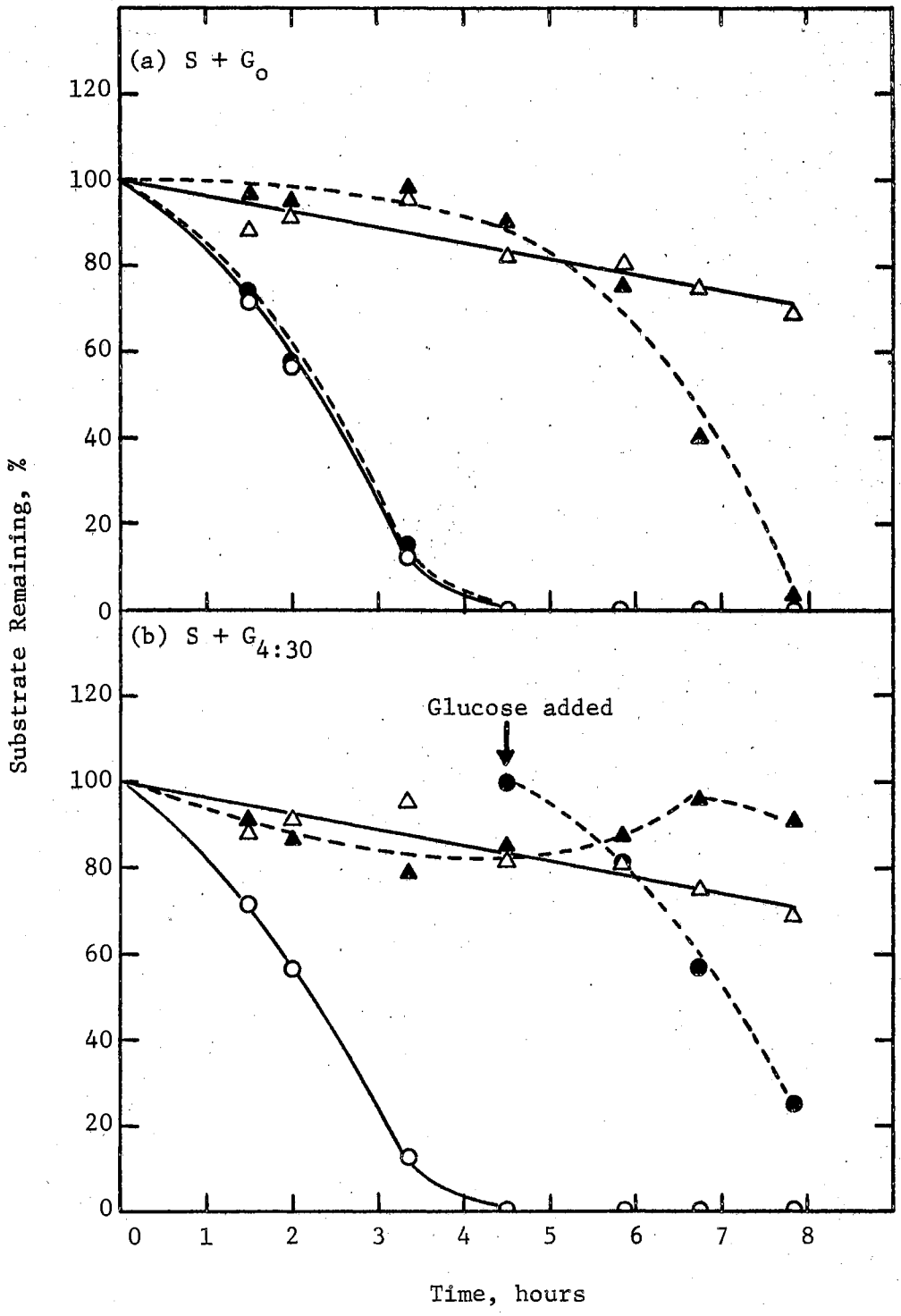

Substrate Remaining, %
130
120
110
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
9
Time, hours

Figure 29. Effect of Glucose On Utilization of Sorbitol by E. coli 45, Old Cells Adapted to Sorbitol in Continuous Flow.

The experiment was performed as described in the text. Glucose was added: (a) at zero time, (b) after 4 hours, 30 minutes. Glucose concentration in the control (○), in the mixtures (●); sorbitol concentration in the control (△), in the mixtures (▲).


(a) S + G_o
(b) S + $G_{4:30}$
Glucose added
Substrate Remaining, %
Time, hours
0
20
40
60
80
100
120
0
1
2
3
4
5
6
7
8

cent, 45 hours; 16 per cent, 6 hours; 14 per cent, 17 hours; 20 per cent, 10 days. Cells from the chemostat effluent were streaked on EMB-lactose medium and on nutrient agar periodically to check for contamination. A single colony from the last nutrient agar plate was used to inoculate the chemostat for the next experiment.

Figure 30 shows the steady state concentrations of cells and sorbitol at the different dilution rates used. It can be seen that the sorbitol concentration remained constant between 8 and 18 per cent but at 20 per cent, cells were washed out and the sorbitol concentration in the reaction vessel increased. The increased concentration of cells at a pumping rate of 4 per cent was due to the evaporation of the medium, which was approximately as rapid as the inflow rate.

Figure 31 shows calibration data for the pump. Dilution rates could not be calculated or controlled precisely because of variations in the air flow rate which caused variations in the volume of culture retained in the reaction vessel. Approximate dilution rates corresponding to the pumping rates used in growing the cells used in experiments are shown in Table VII. Inflow rates were determined by measuring the volume of the water pumped out by the motor within a measured time interval at a definite pumping rate.

The chemostat was operated at a pumping rate of 20 per cent at 37°C for 6 days; the cells were harvested and used in a substrate removal experiment at 37°C. Figure 32 shows the effect of glucose addition. Sorbitol metabolism was completely inhibited after the introduction of glucose, until glucose had been exhausted.

Table VIII shows the optical density data for this set of experiments.

Figure 30. Steady State Concentrations of Cells and Substrate.

The experiment was performed as described in the text. Cell density (○), sorbitol concentration (△).

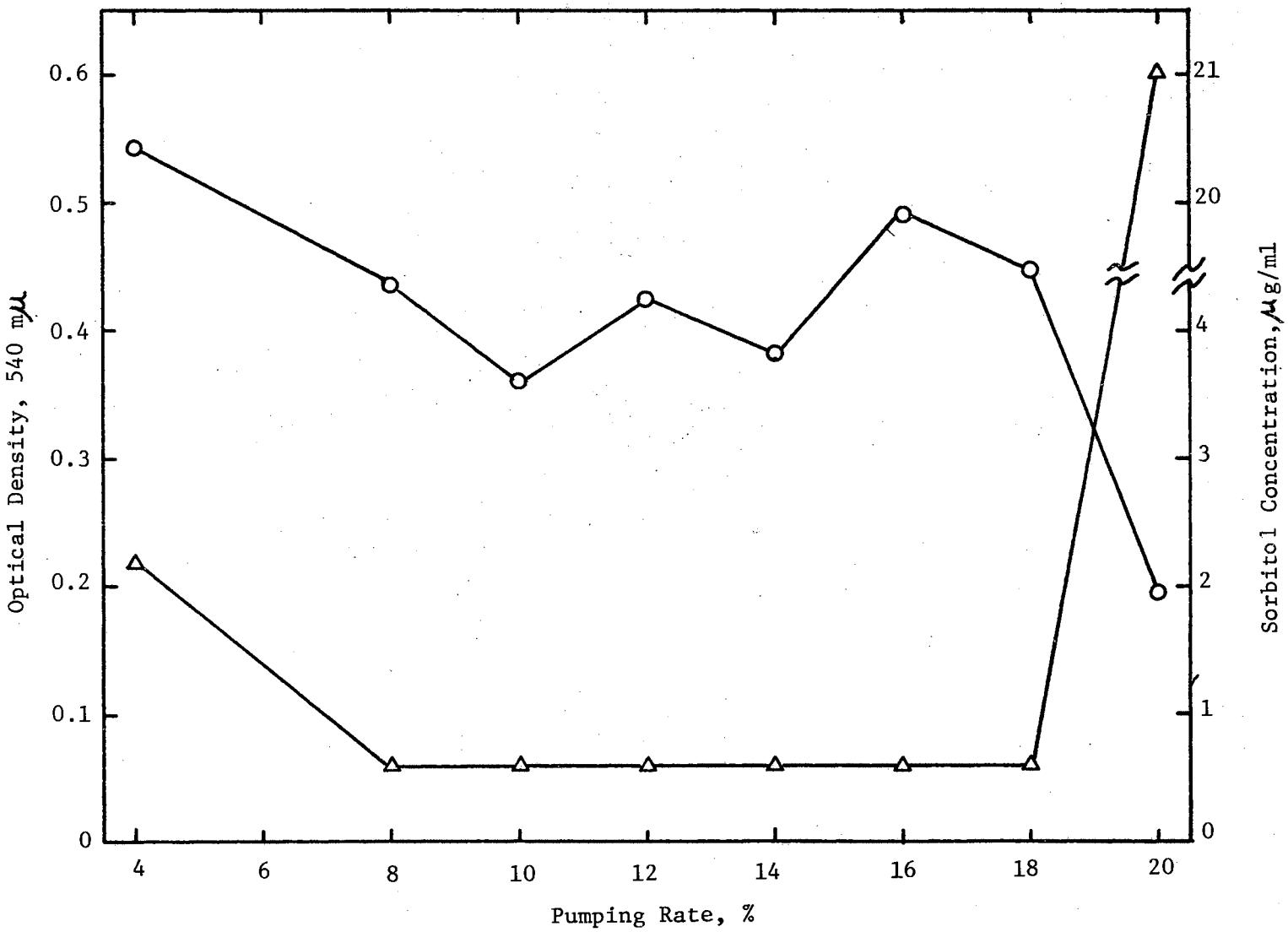

Optical Density, 540 mμ
Sorbitol Concentration, μg/ml
Pumping Rate, %
0
0.1
0.2
0.3
0.4
0.5
0.6
1
2
3
4
20
21
4
6
8
10
12
14
16
18
20

Figure 31. Calibration of Feed Pump for Chemostat.

Rotation of pump, turns per 5 minutes (○); flow rate, ml per min. (△).

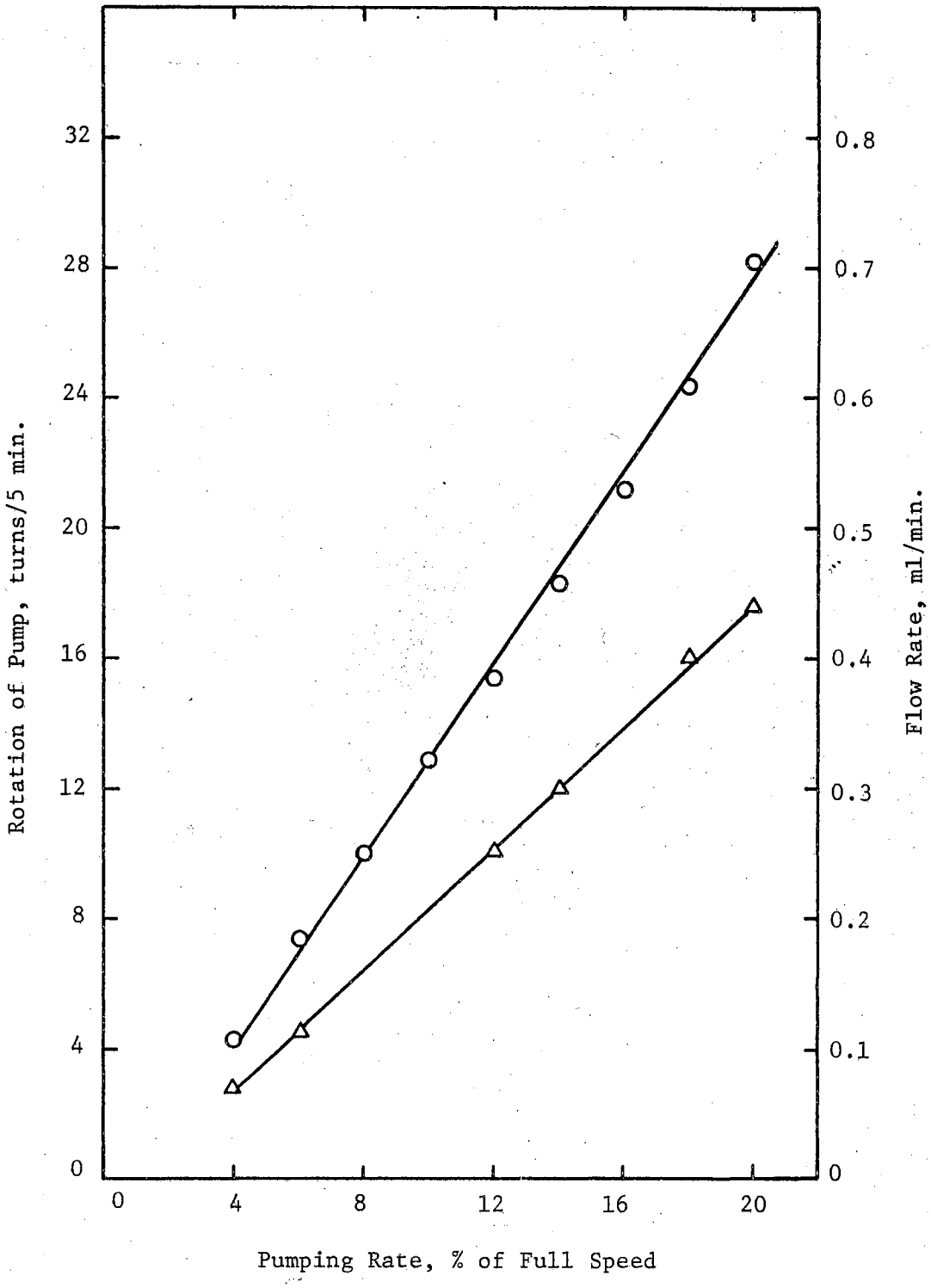

Rotation of Pump, turns/5 min.
Flow Rate, ml/min.
Pumping Rate, % of Full Speed
0
4
8
12
16
20
24
28
32
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8

TABLE VII

DILUTION RATE OF PUMPING MOTOR

Pumping Rate % of Capacity	Flow Rate (ml/min.)	Dilution Rate (per hour)
4	0.07	0.05
6	0.114	0.08
8	0.16	0.112
12	0.25	0.17
14	0.3	0.21
18	0.4	0.28
20	0.444	0.31

Experiments were carried out by measuring the volume of the water pumped out by the motor within a definite time at a definite pumping rate.

Figure 32. Effect of Glucose on Utilization of Sorbitol by E. coli 45, Young Cells Adapted to Sorbitol in Continuous Flow.

The experiment was performed as described in the text. Glucose was added: (a) at zero time, (b) after two hours. Glucose concentration in the control (○), in the mixtures (●); sorbitol concentration in the control (△), in the mixtures (▲).

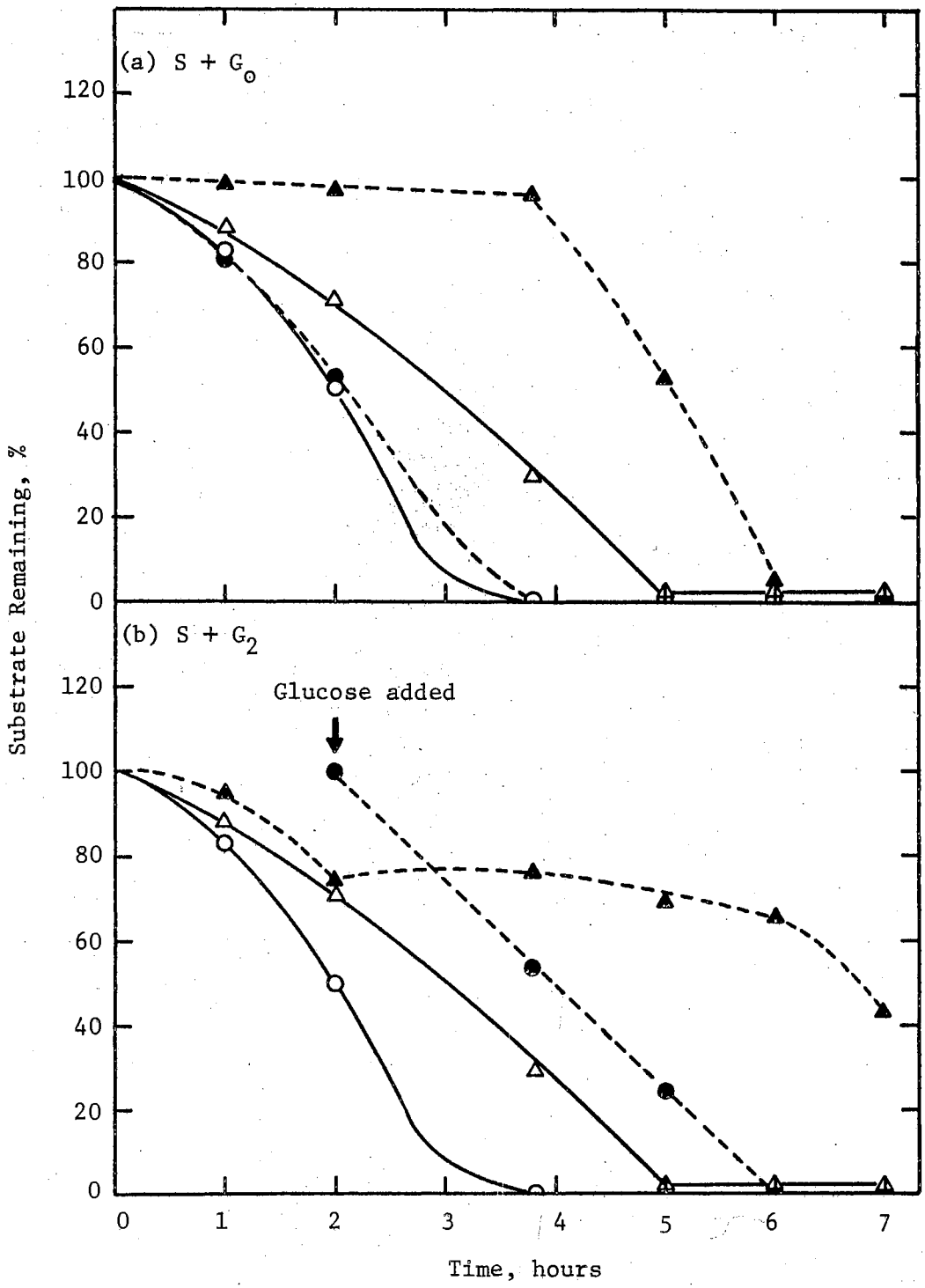

(a) S + G_o
(b) S + G_2
Glucose added
Substrate Remaining, %
Time, hours
0
20
40
60
80
100
120
1
2
3
4
5
6
7

TABLE VIII

OPTICAL DENSITIES FOR ESCHERICHIA COLI 45 IN THE EXPERIMENTS USING CELLS GROWN IN CONTINUOUS FLOW SYSTEMS

Figure	Initial O. D.	Final O. D.			
		Control	Glucose Control	1st Additions	2nd
28	.351	.653	.634	.763	
29	.486	.520	.694	.810	.688
32	.119	.475	.441	.634	.620

Experiments were carried out as described in the text. Cell densities are expressed as optical density readings at 540 mμ.

CHAPTER IV

DISCUSSION

Flask Cultures

Substrate Interaction in Young Cell Populations

This portion of the studies was carried out by introducing a new carbon source while the cells were actively metabolizing the carbon source on which they had been grown. The data obtained in these studies indicate that the pathways for these compounds are subject to feed-back inhibition. All the carbon sources used are metabolized via different pathways, but all have common intermediates. It is postulated that one or more metabolic product can accumulate and can rapidly inhibit the activity, via allosteric inhibition, of the first enzyme responsible for breakdown of the less rapidly metabolized carbon source.

It can be seen from the results that the inhibitory effect between compounds does not seem to be limited to specific combinations of a few compounds and that there are varying degrees of feedback inhibition which are possible, depending upon the compounds employed.

A general metabolic flow chart for carbohydrates and sugar alcohols is shown in Figure 33. Oxidation of sorbitol leads directly to hexose, which is further phosphorylated and enters the oxidative pathway as phosphorylated hexose. Another pathway involves initial phosphorylation of sorbitol which is then oxidized, thus also yielding the phos-

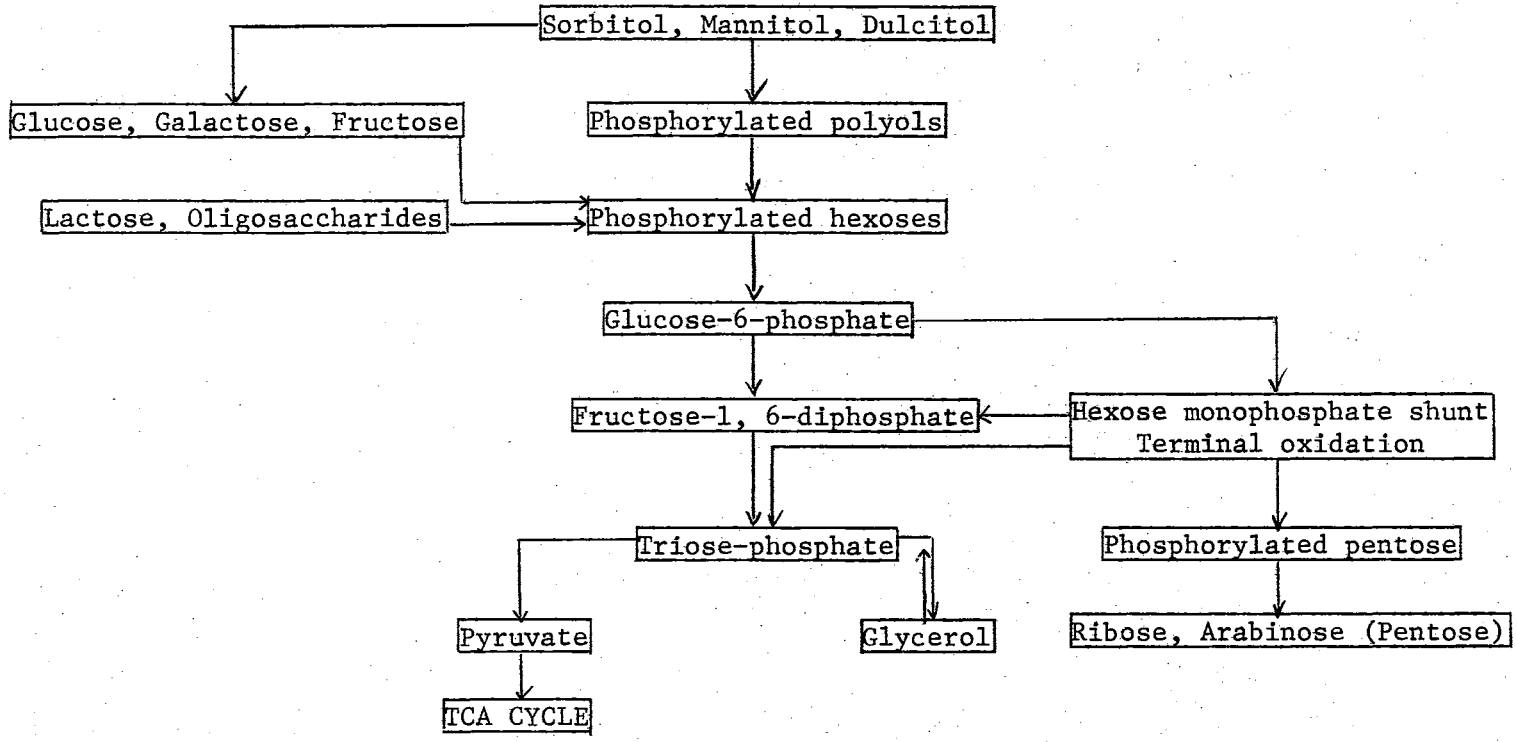


Figure 33. Generalized Metabolic Flow Chart for Various Carbohydrates and Related Sugar Alcohols.

phorylated hexose (Touster and Shaw 1962). Glucose also enters the same metabolic pathway at the level of the phosphorylated hexoses. Hence, it may be that sorbitol and hexoses are metabolized via closely related pathways which may yield common intermediary metabolites. In the case of glucose addition during metabolism of sorbitol by E. coli 45, there was an immediate and complete blockage of sorbitol utilization (Figure 2). The data afford evidence of the existence of a rapid metabolic control system in a catabolic pathway.

In Achromobacter sp., the sorbitol concentration in the medium increased after the addition of glucose (Figure 5). This was not due to the formation of an intermediate, such as glycerol or another polyalcohol, during glucose metabolism (Figure 8). In the histidine-glucose experiment (Figure 9), the rapid metabolism of glucose did not cause the excretion of accumulated histidine, nor was the metabolism of histidine affected as drastically as that of sorbitol by the addition of glucose. Similar observations have been made, however, with a different type of compound which tend to support the conclusion that it is the substrate itself, and not an intermediate, which is excreted. M. Katar (personal communication) found that the concentration of pentose (orcinol-positive material) increased when glucose was added to Salmonella typhimurium metabolizing arabinose.

The addition of a second sugar to cells which had reached a diffusion equilibrium with a nonmetabolizable sugar has been shown to cause the first sugar to leave the cell against a concentration gradient. This phenomenon, known as uphill transport induced by counterflow, has been described by Rosenberg and Wilbrandt (1957), Cirillo and Young (1964), Crocken and Tatum (1966), and Moses and Prevost (1966). It

is interesting to note that with young cells which were able to metabolize sorbitol fairly rapidly, counterflow did not occur immediately upon addition of glucose unless a large amount of sorbitol had already been removed (Figures 5 and 6). In Figures 5a, 6a, 6b and 6c, it would appear that glucose affected the metabolism of sorbitol so that it accumulated without being metabolized, and the non-metabolized substrate was then excreted as more glucose was taken up.

The enzymes of the glycerol pathway appeared to be inducible and also subject to inhibition during the metabolism of glucose. Glucose was effective in preventing the dissimilation of glycerol in both Escherichia coli and Achromobacter sp. The inhibitory effect of added glucose on the uptake of glycerol by induced cells possibly implies that the enzyme glycerol kinase, which converts glycerol into α-glycerol phosphate, had been rendered inactive by fructose-1,6-diphosphate formed from glucose. This inhibitory mechanism was reported by Zwaig and Lin (1966) in a mutant of E. coli. The uptake of glycerol would continue at a constant rate in the presence of the active kinase. Very low levels of glucose were seen to prevent the use of glycerol.

When arabinose-grown cells were exposed to glucose while metabolizing arabinose, it was found that there was considerable inhibition in E. coli 45 if glucose was added at zero or one hour, but there was only slight inhibition with addition at 2 or 3 hours. Glucose was highly inhibitory to arabinose metabolism in Achromobacter sp. whether added early or late. Arabinose utilization was decreased by 90 per cent in the presence of glucose. When ribose-grown cells were exposed to glucose, there was only partial inhibition of ribose utilization. It is interesting that these two pentoses are affected differently. The

specific intermediate required for feedback control may be different for the pathways for these two compounds. Both ribose and arabinose are known to be metabolized via the hexose monophosphate shunt but ribose can enter the pathway more readily than can arabinose. Since ribose is an essential metabolite, it might not be advantageous for the cell to possess a mechanism for preventing its uptake.

Mechanism of Inhibition - Involvement of Permease

These and previous studies have shown that the introduction of glucose into the medium will decrease the rate of removal of a carbon source already present. This can be explained in two ways. First, the function of the enzymes required for the dissimilation of the first compound are inhibited by glucose itself or by a metabolic intermediate. When the cell is saturated with a non-metabolized sugar, no more is removed from the medium. Second, the uptake of the first compound is prevented or decreased by simple competition for a common permease. In the first explanation, the permease could also be involved, as the first enzyme specifically responsible for utilization of the substrate. The distinction, so far as mechanism is concerned, would be between competition for the same transport system and inhibition of the permease by a metabolite accumulated during rapid catabolism of glucose.

Cohn and Monod (1953) suggested that glucose could prevent the entrance of inducer molecules into the cells. Horecker et al (1960a and 1960b) suggested that glucose may compete at the level of a common transporter substance, even though the entrance permeases catalyzing uptake of the external sugars were specific. However, studies of competitive inhibition of uptake have generally shown that the degree of

inhibition decreases with decreasing ratios of glucose to the second sugar. Even at high ratios of glucose to the second sugar, inhibition is not complete. Crocken and Tatum (1967) studied the effect of glucose on sorbose uptake and found that sorbose uptake was competitively inhibited by glucose but the degree of inhibition decreased with increasing glucose concentration; i.e., the degree of inhibition of uptake of 2.0 per cent sorbose at 0.2 per cent glucose concentration was 26 per cent and after increasing glucose concentration to 2.0 per cent, the degree of inhibition increased only about three-fold (82 per cent).

The data obtained in the present study are quite different. The experiments shown in Figures 13, 14 and 15 were designed to check for competitive inhibition. Figure 13 shows that the inhibition of sorbitol removal does not decrease with decreasing concentrations of glucose. Actually, the lowest concentration of glucose (0.02 per cent) was apparently the most inhibitory. The experiment shown in Figure 14 was rather inconclusive since glucose was removed so rapidly that the sampling time did not allow detection of inhibition. In the next experiment (Figure 15), the glucose (and therefore the time required for its removal) was held constant and glycerol concentration was varied. There was no apparent relationship between the degree of inhibition and the ratio of the two compounds.

Actually, the same information is incorporated in each experiment, since the glucose concentration is continually decreasing as it is metabolized and the ratio of the two compounds is also continually changing. If the glucose and glycerol simply compete for a single permease, the glycerol uptake should become increasingly apparent as the level of glucose decreases. It may be seen from Figure 15, and others,

that this did not occur.

Additional evidence that competition for a common permease cannot explain the inhibition observed in these studies lies in the fact that glycerol, which is subject to the inhibition, does not enter by active transport in organisms where this has been studied. Hayashi and Lin (1965) reported that the cells of E. coli K 12 do not possess an active transport system for glycerol and that, even at very low concentrations, the entry of glycerol by free diffusion is not rate-limiting for growth. Cowen (1968) also showed that glycerol was not transported into the cell at a rate greater than that of free diffusion even in fully-induced cells of Pseudomonas aeruginosa. In this organism, experiments identical to those carried out in the present study, using glucose and glycerol, resulted in the same type of inhibition. Therefore, it does not seem unreasonable to assume that there is no active transport mechanism for glycerol in Achromobacter sp. Therefore, glucose could not compete for the same permease with glycerol.

The closely related hexose galactose was tested for an inhibitory effect similar to that of glucose. For these experiments, strain W3110 of E. coli was obtained from Dr. M. L. Morse, along with several mutants blocked in different steps of the catabolic pathway for galactose. It was hoped that comparison of the effect of galactose on metabolism of other sugars by the wild type and mutants would aid in study of the mechanism of the inhibition. However, it was found that the wild type used galactose quite slowly. Figures 16 and 17 show that galactose, sorbitol and ribose were utilized at almost equal rates. The addition of galactose to the culture did not cause the inhibition of sorbitol or ribose utilization. There was certainly no competition for a permease,

although this might have been expected if glucose acted through this mechanism since glucose had been reported to compete for the galactose permease (Adhya and Echols, 1966), and glucose, galactose, sorbitol and ribose might therefore share a common step in transport.

Mandelstam (1962) showed that the ability of different carbon sources to repress synthesis of inducible enzymes varied with the rate at which they were metabolized and with the growth rate of the cells. Thimann (1963) has stated that the repressive effect of a compound seems to be dependent on the rate at which its intermediary catabolites are being synthesized as well as being utilized. If it is assumed that feedback inhibition of a catabolic pathway is also dependent upon accumulation of intermediates, the same factors should affect both repression and inhibition. Galactose may accumulate a lower level of metabolites since it is utilized much more slowly, and thus may not allow the build-up of the metabolites to a critical level that would set the control mechanism into full operation.

Aged Cells

Studies with heterogeneous populations had shown that the responses of old cells to addition of a second carbon source such as glucose were quite different from those of the young cells. Thimann (1963) pointed out that the age of a bacterial population might involve changes in the permeability of the cell membrane to a substrate and noted that very limited knowledge was available with regard to the effects of this physiological phenomenon. Glucose, which is normally considered to be readily utilizable by almost all microbial species since the enzymes required for oxidative assimilation of glucose are thought to be cons-

titutive, was not readily degradable by old cells in heterogeneous populations. It was thought possible that glucose permease, required by the cells for glucose uptake, or an initial enzyme step required to bring glucose into the Embden-Meyerhof pathway was absent in the old cells and had to be induced.

Various methods of aging cells were employed in the present study to determine whether the loss of sensitivity to glucose was a typical effect of aging. In the first experiment, which was shown in Figure 19, sorbitol utilization was faster than glucose utilization in control flasks with six-day old cells; however, in glucose-sorbitol mixtures, the glucose effect is obvious. In order to observe the substrate interactions at daily intervals during aging of cells, substrate removal experiments were run every day using cells from identical flasks started at the same time (Figures 20-22). Sorbitol metabolism in E. coli 45 aged without yeast extract became increasingly slower as the cells aged and was inhibited by the introduction of glucose from the first day to the fourth day. On the fifth day, sorbitol utilization was very slow, so that it was difficult to say whether sorbitol removal was inhibited or not. When E. coli 45 was aged with daily additions of yeast extract as well as sorbitol, or when Achromobacter sp. was aged, two interesting changes occurred (Figures 23-24 and 25-27). The rate at which sorbitol was metabolized decreased daily in both cultures. Since the cell density increased throughout the aging period, more cells were involved in each consecutive experiment and therefore the decrease in rate of sorbitol metabolism was even greater than is apparent from the figures. The second consistent change which occurred in both cultures was a greatly increased tendency toward counterflow of sorbitol on addition of glucose.

This may indicate a decrease not only in uptake rate for sorbitol but also in its rate of conversion inside the cell.

In all these studies, the glucose effect was still apparent and the cells never lost their capability for rapid metabolism of glucose; i.e., the glucose degradative enzymes were constitutive even in aged cells, rather than inducible. It is possible that cell age was not as great as that of the heterogeneous populations studied. However, it is more likely that, in aging a heterogeneous population, species are selected which can, or do, cease to manufacture unused enzymes, such as those needed for glucose metabolism if glucose is not present.

Continuous Flow Studies

Since the cell density continually increased during aging of cultures in flasks, it was impossible to determine the real age of the cells in the culture. Therefore, it was decided to use a chemostat to obtain cells of known mean generation time. According to the steady state concept, the growth rate of a culture would be equal to the dilution rate, provided that the system is maintained under steady state conditions. Thus, the population of the system operated in a steady state would always be in a constant logarithmic growth phase, regardless of the detention period employed, until the dilution rate exceeds the maximum growth rate.

In this portion of the studies using the continuous flow system, the steady state concentrations of bacteria and substrate in the reaction vessel were varied by changing flow rate and temperature, keeping the concentration of carbon source in the inflow constant (Herbert, Elsworth and Telling, 1956). The maximum generation time attainable was limited

by the apparatus itself, since at the lowest dilution rate used evaporation balanced inflow rate even though the inflowing air was bubbled through water. The minimum generation time attainable without dilute-out was approximately 3.2 hours for the sorbitol minimal medium at 37°C (Figures 30 and 31 and Table VII). The physiological characteristics of these cells were similar to those observed in the young cell populations which were developed in the previous flask studies.

E. coli 45, grown on sorbitol at room temperature, still can use sorbitol and glucose very rapidly after transfer to a flask culture at 37°C (Figure 28). It is obvious that sorbitol was removed at a somewhat slower rate than was glucose, although the culture had been maintained on sorbitol. No adaptation to glucose was required. In the experiment shown in Figure 29, sorbitol utilization was very slow, but there was still some effect on sorbitol metabolism when glucose was added after 4.5 hours. These were the oldest cells used in the chemostat studies, although their age could not be determined due to evaporation of medium. Figure 32 shows the typical glucose effect obtained with young cells. These cells were growing at a rate of 0.31/hour. Glucose addition caused the complete inhibition of sorbitol utilization; after glucose had been exhausted sorbitol was metabolized again.

Although, the maximum range of growth rates attainable by the cells was probably not attained in these experiments, growth rates differing by at least five-fold were employed. Even cells grown at the slowest possible rate (Figure 29) retained the ability to use glucose quite rapidly but used sorbitol much more slowly. These results are in agreement with those obtained with cells aged in flask cultures. Also in agreement with the previous results, glucose inhibited utilization of

sorbitol by old or young cells, although the effect was more striking with young cells simply because of the more rapid utilization by non-inhibited cultures.

CHAPTER V

SUMMARY AND CONCLUSIONS

The ability of Escherichia coli strain 45 and Achromobacter sp. to use various carbon sources as sole substrates for growth was studied. Combinations of these substrates were then used to study substrate interactions. The addition of glucose to a growth medium was shown to exert rapid inhibition of the utilization of the first substrate which either young or aged cells were actively metabolizing. The degree of inhibition varied with the compound employed.

Two possible explanations for this rapid inhibition were suggested. The first is feedback inhibition due to an accumulated intermediate. Second is a simple competition for a common permease. Data obtained in these studies indicate that glucose and sorbitol do not compete for a single permease, since the degree of inhibition is not proportional to the concentration of glucose. In addition, identical inhibitory effects were observed when glucose was added to cultures metabolizing glycerol, which enters the cell by diffusion rather than by active transport.

In many experiments in which glucose was added to cells metabolizing sorbitol, it was found that the addition of glucose causes the counterflow of sorbitol. This effect was exaggerated with older cells which metabolized sorbitol slowly.

Escherichia coli strain 45 could be maintained in the steady state at 37°C in a chemostat fed minimal medium containing 0.2 per cent

sorbitol at dilution rates of 0.112 $hour^{-1}$ to 0.28 $hour^{-1}$. At a dilution rate of 0.31 $hour^{-1}$, corresponding to a mean generation time of 3.2 hours, cells began to dilute out. Cells grown at the lowest attainable growth rate in the chemostat were still sensitive to glucose; i.e., addition of glucose to the sorbitol medium inhibited utilization of sorbitol. Similar results were obtained with both E. coli 45 and Achromobacter sp. using cells aged by prolonged incubation in sorbitol medium. In all cases, older cells showed greatly decreased ability to utilize sorbitol but retained the ability to use glucose rapidly.

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